The Role of Quality DNA in Molecular Plant Taxonomy and Phylogenetic Studies

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Abstract- Traditional approach in plant taxonomy for characterization, identification, classification and nomenclature of plants is based on phenotypic similarities and differences of plants.

Phenotypic traits of plants may vary due to environmental conditions and often create major problems in proper identification. Recent advances in molecular techniques can help plant taxonomist to overcome these problems. Most of the molecular techniques are PCR based and one of the major challenges in molecular taxonomy and phylogenetic study is to extract quality DNA from voucher herbarium specimens, which are stored plant material for taxonomic studies.

We have compared the one-tube extraction method with the traditional Cetyltrimethylammonium bromide (CTAB) method on different herbarium samples and found that the one-tube extraction method gave a good amount of DNA suitable for PCR as compared to CTAB method.

Key words: Molecular Taxonomy, DNA extraction, one tube method, PCR

I.INTRODUCTION

Plant taxonomy and phylogenetic studies helps to understand biodiversity, conservation and proper use of plant resources. For proper identification, classification, nomenclature and as reference data. Plant taxonomist collect a huge number of plant samples and store it in a form of pressed dried material called as herbarium. It is estimated that around 70,000 new species already housed in herbaria are not described yet (Bebber et al., 2010). The systemic phenotypic study of herbarium face the problems of identification errors, lack of communication and coordination (Rouhan G. and Gaudeul M, 2014). Phenotypic characters are highly variable in respect to climatic conditions and often create major problems in proper identification (De and Bandyopadhyay, 2008). Recent advances in molecular techniques have come up with solutions to overcome these problems (Arif et al., 2010). Most of the molecular techniques are PCR based and so the role of quality DNA is the crucial starting point in molecular plant taxonomy and phylogenetic studies.

Molecular plant taxonomist struggle with various issues in DNA extraction from herbarium specimens, old air-dried material that has not been treated with chemical preservatives, high temperatures, or microwaves has the best chance of yielding useful DNA, but to keep specimens free from insect and pest chemical treatment is a must. (Drábková, 2014). The DNA from herbarium specimens is usually highly degraded and fragmented, making its extraction from old tissues particularly difficult. The generally limited success of DNA extraction and the challenges associated with the PCR amplification of highly degraded DNA limited the use of historical specimens for molecular analysis (Xu et al., 2015). Researchers need a robust DNA extraction protocol which can overcome these struggles and give a good quality of template DNA which is high throughput method, cost effective, can yield good quantity of DNA from very less amount of dried specimen which are chemically treated.

In this paper we are presenting a DNA extraction protocol which is an alkaline crude nucleic acid extract which gives a good quantity of DNA applicable for PCR (Wang et al. 1993).The concentration of extraction buffers and the quantity of extract taken for PCR is standardized to give a good template of DNA for PCR. We have compared these One tube Method method with the routine CTAB method as described by *Saghai-Maroof* et al. (1984) with slight modification. DNA was extracted following these two methods and quality of DNA was tested with PCR using plant-specific 28KJ/28C primer combination

(Cullings, 2008). CTAB method works very well for fresh leaf tissue.

The herbarium specimens were collected from J.E.S College, Jalna. The specimens were a collection of leaf samples of trees from College campus in the year 1999. A total of 12 specimens were collected of plants listed as:

S.No	Botanical name	Common name
1	*Acacia Nilotica*	Babul
2	*Azadirachta indica*	Neem
3	*Mangifera indica*	Mango
4	*Bambusa Rotundifora*	Bamboo
5	*Curcuma domestica*	Haldi
6	*Nyctanthas Arbor*	Parijat
7	*Tictona Grandis*	Teak
8	*Buta Monosperma*	Palas
9	*Annona Squamosa*	Custurd apple
10	*Eucalyptus globules*	Nilgiri
11	*Datura stramonium*	Datura
12	*Adathoda Vasica*	Adulsa

Table No. 1: List of herbarium specimens collected for study

II.MATERIAL AND METHOD

A. CTAB PROTOCOL-

Reagents:

a) CTAB Extraction buffer: 100m M Tris-HCl, 20 mM EDTA (ethylenediaminetetraacetic acid) and 1.4M NaCl, pH=8.0 and 2% CTAB (wv-1 hexadecyltrimethylammonium bromide), 2% 2-mercaptoethanol, 1% PVP (polyvinylpyrrolidone pH =8.0)

b) Chloroform: Isoamyl alcohol (C: I): 24:1(v/v), equilibrated to pH = 8.0

c) Isopropanol

d) Ethanol (70%)

e) TE buffer: Tris-HCl 10mM, 1mM EDTA, pH=8.0

CTAB method:

100 mg of leaf sample from each herbaria were taken in 1.5ml centrifuge tube, to it 500 µl of CTAB extraction buffer was added and samples were crushed using mini pestles. Crushed samples were kept at 60^0C water bath for 30 minutes, tubes were taken out of water bath and to it 500 µl of Chloroform: Isoamyl alcohol (24:1) was added, the contained was mixed properly by inverting the tubes. Cell debris get precipitated in the lower chloroform: Isoamyl layer so to separate the cell debris the tubes were centrifuged at 12000 rpm for 10 minutes in micro- centrifuge (REMI RM-12C). The upper supernatant was collected carefully in new 1.5 ml centrifuge tube and to it 0.6 Volume of Isopropanol was added, mixed by inverting the tubes till Nucleic acid gets precipitated. Nucleic acid is collected as a pellet at the bottom of the tube by centrifuging the tubes at 12000rpm for 10 minutes and the supernatant was discarded. The Nucleic acid pellet was washed by 70% Ethanol and then air dried for 5-6 hours at room temperature. Once the pellet is dried it is re-suspended in 100 µl of TE buffer.

B. ONE TUBE METHOD

a) 0.5 M Sodium hydroxide - NAOH

b) 0.5 M Tris-Cl

c) 2% PVP -polyvinylpyrrolidone pH =8.0

1mg of leaf sample from each herbaria were taken in 2 ml centrifuge tube and crushed in a solution of 50 µl of 0.5 M NAOH and 50 µl of 2% PVP. Due to the high concentration of alkaline pH of NAOH nuclear DNA gets released in the solution. PVP helps in preventing DNA damage caused by phenolic compounds and cell enzymes. To stop the lysis process 900 µl 0.5M Tris-Cl was added and mixed the solution by inverting the tubes several times.

C. PCR REACTION

Reagents:

a) 10X Dream Taq buffer with 20 mM $MgCl_2$ (Thermo Scientific)

b) 5U Dream Taq Polymerase (Thermo Scientific)

c) 28KJ/28C primers

d) 10 mM dNTP (Thermo Scientific)

e) PCR grade water (Hi-media)

f) 2% Agarose gel in 1X Tris Borate EDTA buffer (TBE)

g) Molecular Marker- (M) 100bp –(Chromous Biotech)

PCR reaction Method:

DNA samples extracted using protocol A and B were tested with plant specific 28KJ/28C primer combination. The PCR reactions were performed in 0.2 ml reaction tubes using 1 U Dream Taq DNA polymerase, 0.2 mM dNTPs, and 1 X Dream Taq buffer in 25 µl reaction volume. The sequence of samples was as listed in Table NO.1 with plant positive control DNA extracted from fresh leaf sample using CTAB method and a No template control as negative control for PCR reaction. The 2 µl of template DNA from A. CTAB protocol was taken and 0.5 µl of template DNA from B. One tube Method was taken. The Thermo- cycler of Applied Biosystems by Thermo fisher Scientific VERITI was programmed as (2 min 94^0C, 35 cycles with 30 sec 94^0C, 30 sec 50^0C, 1 min 72^0C and final extension step of 5 min at 72^0C) to perform the amplification of plant specific amplicon. The amplified products were separated on 2% Agarose gel in 1X TBE buffer.

III.RESULT AND DISCUSSION

The plant specific 28KJ/28C primers combination gave desired amplicon with template DNA from B. One tube Method in all the 12 herbarium samples. The desired amplicon matched amplification size of positive control. Whereas the DNA extracted using A. CTAB protocol was not suitable for PCR reaction, no amplification was seen in herbarium samples but the positive control worked.

The specimen taken for both the protocol was from the same voucher samples but still there was difference in quality of DNA. The DNA extracted with One tube Method was a crude extract, the amount of template taken had minimum contaminants which did not hindered the PCR reaction. The template DNA was sufficient to give desired amplicon. So we can conclude that the One tube Method is a good protocol with vast applicability for herbarium specimens, as all the samples from different families had worked well. The protocol is easy to handle, cost effective, with the use of minimum chemicals and lab equipment's, a good quality of DNA is extracted which can be used for further molecular taxonomic studies.

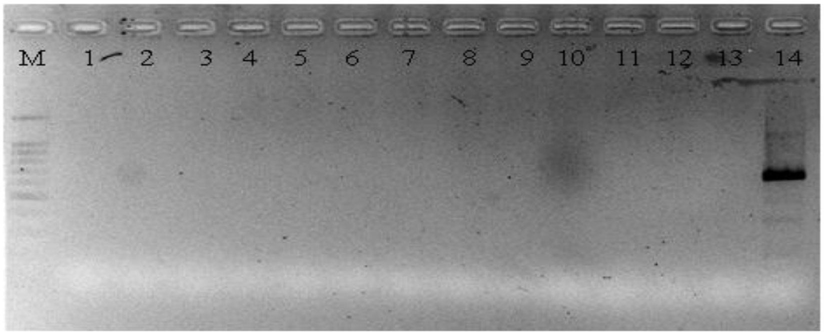

Figure 1. Gel picture PCR reaction with A. CTAB protocol. Lanes 1 to 12 as listed in table no. 1, Lane 13 No template control, Lane 14. Positive control.

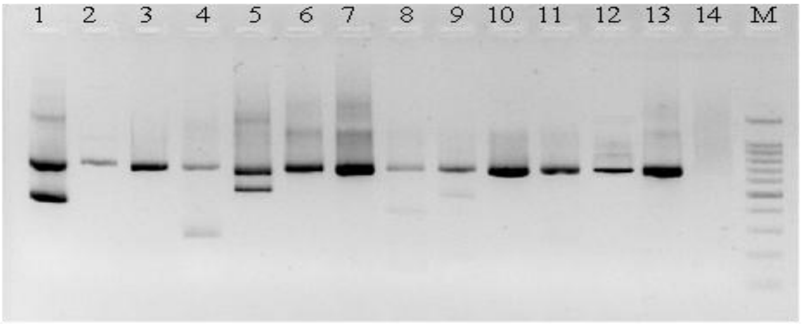

Figure 1. Gel picture PCR reaction with B. One tube Method protocol. Lanes 1 to 12 as listed in table no. 1, Lane 13. Positive control, Lane 14 No template control

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REFERENCE

[1] Ibrahim A. Arif, Mohammad A. Bakir, Haseeb A. Khan, Ahmad H. Al Farhan, Ali A. Al Homaidan, Ali H. Bahkali, Mohammad Al Sadoon, Mohammad Shobrak. A brief review of molecular techniques to assess plant diversity. Int J Mol Sci. 2010; 11(5): 2079–2096. Published online 2010 May 10. doi: 10.3390/ijms11052079.

[2] Bebber, Daniel & Carine, Mark & Wood, John & Davey, Alex & Harris, David & Prance, G.T. & Davidse, Gerrit & Paige, Jay & Pennington, Terry & Robson, Norman & Scotland, Robert. (2010). Herbaria as a major frontier for species discovery. Proceedings of the National Academy of Sciences of the United States of America. 107. 22169-71. 10.1073/pnas.1011841108.

[3] Cullings, Ken. (2008). Design and testing of a plant-specific PCR primer for ecological and evolutionary studies. Molecular Ecology. 1. 233 - 240. 10.1111/j.1365-294X.1992.tb00182.x.

[4] De S, Bandyopadhyay S. Molecular Taxonomy: An Approach Based on Molecular Markers. Science and Culture 2008, Vol-74, 397-496. http://www.scienceandculture-isna.org/20081213.htm

[5] Rouhan G, Gaudeul M. Plant taxonomy: a historical perspective, current challenges, and perspectives. *Methods Mol Biol* 2014;1115:1-37. [PMID: 24415467 DOI: 10.1007/978-1-62703-767-9_1

[6] Saghai-Maroof, M.A., Soliman, K.M., Jorgensen, R.A. and Allard, R.W. 1984. Ribosomal DNA spacer length polymorphism in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc. Natl. Acad. Sci. USA 81: 8014-8018.

[7] Wang, H., Qi, M. Q., and Cutler, A. J. 1993. A simple method of preparing plant samples for PCR. Nucleic Acids Res. 21:4153-4154.

[8] Xu, C., Dong W., Shi S., Cheng T., Li C., Liu Y., Wu P., et al. 2015. Accelerating plant DNA barcode reference library construction using herbarium specimens: Improved experimental techniques. *Molecular Ecology Resources* 15: 1366–1374. [PubMed] [Google Scholar] [Ref list]

[9] Záveská Drábková, Lenka. (2014). DNA Extraction from Herbarium Specimens. Methods in molecular biology (Clifton, N.J.). 1115. 69-84. 10.1007/978-1-62703-767-9_4.