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Rapid seed DNA extraction for species identification and diversity analysis of Pumpkin

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Abstract

Cucurbita moschata an economically important species of the family Cucurbitaceae, shows high variability in fruit characteristics. A standardized DNA isolation protocol has been developed from dried pumpkin seeds for polymerase chain reaction for species identification and diversity analysis. Higher concentration of polysaccharides and polyphenols in pumpkin seeds interferes with DNA during its isolation resulting in no PCR products. Good quality DNA, with no coloured pigments and contaminants was isolated from sun dried pumpkin seeds with modified CTAB buffer protocol without using liquid nitrogen. The average DNA concentration obtained was 63.9µg/gm with purity ranging between 1.66 to 1.85. The isolated DNA was successfully amplified using barcoding (rbcL), RAPD and SSR primers. The quantity and quality of the DNA isolated by this method was high enough to perform more than 150 PCR reactions. The species identify for *Cucurbita moschata* was also confirmed through sequencing and NCBI BLASTn analysis of bracoding primer (rbcL) product using isolated DNA. On the basis of UPGMA analysis, 14 pumpkin genotypes were categorized into two broad clusters. Broad cluster I and II comprised of one genotype (NEHUP8) and 13 independent genotypes respectively. The major cluster-I comprised of two genotypes *viz.* NEHUP1 and NEHUP14 with a genetic similarity percent of 0.58 approximately. The major cluster-II was sub divided into three minor clusters. This modified DNA isolation protocol may be adequate for isolating high-molecular weight DNA from other cucurbitaceous species containing large amounts of secondary metabolites.

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Introduction

Cucurbita moschata Duch. (2n= 2x= 40) of Cucurbitaceae family commonly known as pumpkin is a very popular vegetable in many tropical and sub tropical countries. It is highly nutritious and is advantageous over other vegetables as its fruit can be stored for several months before consumption. The plant is cultivated for its young shoots, fleshy edible flowers, fruits as well as for seeds. It has also been known for its use in traditional medicine as ant diabetic, antihypertensive, antitumor, immunomodulator, antibacterial, antihyperc holesterolemic and anti-inflammatory activities.

There is a tremendous genetic diversity within the crops of cucurbitaceae family and the knowledge of genetic diversity in a crop species is fundamental to its improvement. For diversity analysis morphological traits have many limitations, including low polymorphism, low heritability, and late expression. Now a-daysvery powerful PCR-based techniques such as like Randomly Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR) analysis have emerged which are very fast, reliable and require minimal amount of tissue to evaluate genetic diversity. Day by day development of such new and specific type of markers make their importance in understanding the genomic variability and the diversity between the same as well as different species of the plants (Kumar *et al.*,2009).

In molecular studies for diversity analysis DNA plays a significant role. The use of complementary techniques such as DNA sequences may enhance taxonomic and systematic studies (Herbert *et al.,* 2003). But for studying the molecular systematic of any organism, high quality DNA is prerequisite (Schander and Halanych, 2003). A good quality of template DNA is also required in Polymerase chain reaction (PCR) for getting a successful result and it ultimately depends on quick and inexpensive method of DNA isolation.

Again the yield of isolated DNA varies considerably among taxa and should not be extrapolated from one organism to another (Dillon *et al.,* 1996; Dawson *et al.,* 1998; Mtambo *et al.,* 2006).

However, the integrity of the isolated DNA will vary according to the organism, preservation conditions, time of storage and DNA isolation method (Salgado *et al.,* 2007). In plant science, DNA is generally isolated from leaf tissue. But the chance of contamination of isolated DNA with secondary metabolites like poly-saccharides, proteins such as tannins, alkaloids, and polyphenols is more when DNA is being isolated from seed tissue.

Several DNA isolation protocols have been optimized and were used in various combinations to isolate quality DNA from plants for analyses (Dellaporta *et al.,* 1983; Doyle and Doyle, 1990; Suman *et al.,* 1999; Shah *et al.,* 2000; Warude *et al.,* 2003; Sarwat *et al.,* 2006; Deshmukh *et al.,* 2007). Most of this DNA isolation protocols uses liquid nitrogen for obtaining good quality DNA. But liquid nitrogen is not always easily accessible especially in developing parts of the world. It is also difficult to isolate and purify high quality DNA from the seeds of some vegetables like pumpkin because of the presence of different polysaccharides and polyphenols which interferes with the quality and yield of the DNA being isolated. Secondary metabolite interfere by precipitating along with the DNA, thus degrades its quality and reduces yield and also generates no amplified products upon amplification using Polymerase chain reaction (Katterman and Shattuck, 1983; Sarwat *et al.,* 2006).

The aim of this investigation was to develop a standardized DNA isolation protocol to isolate good quality DNA from pumpkin seeds without the use of liquid nitrogen for amplification in polymerase chain reaction (PCR) using bar-coding (rbcL), RAPD and SSR primers for species identification and genetic diversity analyses.

Materials and methods

Plant material

Mature fruits of pumpkin (*Cucurbita moschata* Duch.) were collected from different parts of Garo Hills of Meghalaya, India (Table 1) during the year 2014-2015. Seeds were then extracted from fully mature fruits. Finally the sun- dried and shade-dried seeds were used to isolate the DNA using a modified CTAB (cetyl trimethyl ammonium bromide) method without the use of liquid nitrogen.

Table 1. Places of collection of Pumpkin (*Cucurbita moschata* Duch.) germplasms from different parts of Garo Hills of Meghalaya, India.

Sl No	Accession No	Place of Collection	Fruit Shape	Sl No	Accession No	Place of Collection	Fruit Shape
1	NEHUP 1	Rongsak	Disk	8	NEHUP 8	Chokpot	Pyriform
2	NEHUP 2	Rongram	Pyriform	9	NEHUP 9	Purakhasia	Disk
3	NEHUP 3	Dalu	Flattened	10	NEHUP 10	Resubelpara	Globular
4	NEHUP 4	Baghmara	Oval	11	NEHUP 11	Dadenggre	Elongate
5	NEHUP 5	Jengjal	Oval	12	NEHUP 12	Anogre	Oval
6	NEHUP 6	Williamnagar	Flattened	13	NEHUP 13	Garobadha	Globular
7	NEHUP 7	Gambegre	Oval	14	NEHUP 14	Amindagre	Elongate

Reagents for DNA extraction and purification

Tris HCL, Tris Base, CTAB, PVP, EDTA, NaCl, Sodium acetate, Phenol: Chloroform: isoamyl alcohol (25:24:1), β-mercaptoethanol, HCL, NaOH, Isopropanol, 70% Ethanol, TE buffer, Absolute ethanol, Enzyme (Taq Polymerase, Thermo fisher Inc.), Buffer (Taq DNA Polymerase buffer, Thermo fisher Inc.), Nucleotides (dNTPs, Thermo fisher Inc.), RAPD primers (IDT Inc.), SSR Primers (IDT Inc.), Rnase A (Thermo fisher Inc.), 1X TAE buffer, Agarose and Ethidium bromide.

Modified CTAB DNA isolation buffer

2% CTAB, 2% PVP, 100mM Tris HCL (pH- 8.0), 20mM EDTA (pH- 8.0), 1.4M NaCl, 0.2% β-merca ptoethanol (added just before use).

DNA Isolation

The sun-dried and shade-dried seeds of pumpkin were grinded to fine powder using pre-cooled mortar and pestle and then transferred to a microfuge tube containing the modified isolation buffer. The tubes were then incubated at 65°C for 35-45 mins with 2-3 times inversion during incubation. The tubes were centrifuged at 13,000 rpm for 10 mins. The supernatant were placed in new microfuge tubes. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) were added to each tube and the tubes were then centrifuged at 13,000 rpm for 15 mins. The supernatant were again placed in new microfuge tubes. The same step may be repeated for 3-4 times. An equal volume of absolute ice-cold isopropanol was added and the tubes were centrifuged. The supernatant were discarded and the pellets were washed with 70% ethanol. After washing, the pellets were allowed to air dry for 45-60 mins. Finally the DNA pellets were dissolved in TE buffer and purified. DNA concentrations were measured by running aliquots on 1.5% agarose gel and by reading absorbance at 260 and 280 nm with a double beam UV-VIS spectrophotometer.

DNA Purification

RNase (10mg/ml) was added to the each tube and incubated at 37°C for 1 hr. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and the tubes were centrifuged. The same step may be repeated for 3-4 times. The supernatant were then transferred to new microfuge tubes and 1/10th volume of sodium acetate was added to each tube followed by addition of 2.5 volume of ethanol. The tubes were then kept at -20°C for 30 mins and then centrifuged. The supernatant was discarded and the pellets were washed with 70% ethanol and then air dried. The DNA pellets were dissolved in TE buffer and kept at -20°C in deep freezer.

PCR amplification and gel electrophoresis

PCR-based amplification of the purified DNA of different pumpkin germplasms was carried out using rbc L, RAPD (PPR 1) and SSR (OCM 1 and OCM 2) primers. The total reaction mixture for rbc L, RAPD and SSR primers were 15µl, 25µl and 15µl respectively. The RAPD reaction mixture contained 25ng of template DNA, 1.5U *Taq* DNA polymerase (Thermo fisher Inc.), 10mM dNTPs (Thermo fisher Inc.), 10X *Taq* DNA polymerase buffer (thermo fisher Inc.), 10µM primer (PPR 1). The rbcL and SSR reaction mixture contained 25 ng of template DNA, 0.3 U *Taq* DNA polymerase (Thermo fisher Inc.), 10 mM dNTPs (Thermo fisher Inc.),

10X *Taq* DNA polymerase buffer (Thermo fisher) and 10 µM primer (for rbcL primer: rbc La; for SSR primer: OCM 1 and OCM 2). Amplification of DNA was done using a Bio-Rad thermo cycler (Model: C 1000). The PCR running condition for RAPD (PPR1) primer was: initial denaturation at 95°C for 1 min, followed by 45 cycles of denaturation at 94°C for 1 min, primer annealing at 38.9°C for 1 min and extension at 72°C for 2 mins and the final step of 7 mins at 72°C for final extension. The PCR running condition for SSR (OCM 1 and OCM 2) primer was: initial denaturation at 94°C for 2 mins, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 52.8°C (for OCM 1) and 57.3°C (for OCM 2) for 1 min and extension at 72°C for 1 min, and the final step of 7 mins at 72°C for final extension.

The PCR running condition for rbc L primer was: initial denaturation at 94°C for 1 mins, followed by 35 cycles of denaturation at 94°C for 15 s, primer annealing at 54.5°C for 15 s and extension at 72°C for 30s, and the final step of 7 mins at 72°C for final extension. After the completion of PCR cycles the reaction was stored at 4°C, until it was loaded onto the gel. The PCR products were fractionated on 1.5% agarose gel using 1X TAE buffer containing 10mg/ml ethidium bromide and were visualized under UV light. The gels were then photographed using the Bio-Rad gel doc XR documentation system.

Primer sequences

The primers were synthesized and purchased from IDT (Integrated DNA Technologies). The details of the primers have been mentioned in Table 3.

Table 2. Quantitative estimates of DNA concentration revealed by UV-VIS spectrophotometry.

Sl No	Accession No	Sample Weight (mg)	Conc. µg DNA/g	Λ260/280
1	NEHUP 1	100	82.65	1.70
2	NEHUP 2	100	68.12	1.80
3	NEHUP 3	100	43.72	1.82
4	NEHUP 4	100	52.56	1.77
5	NEHUP 5	100	24.30	1.66
6	NEHUP 6	100	44.68	1.83
7	NEHUP 7	100	10.61	1.75
8	NEHUP 8	100	17.73	1.84
9	NEHUP 9	100	121.27	1.78
10	NEHUP 10	100	111.56	1.66
11	NEHUP 11	100	117.73	1.84
12	NEHUP 12	100	75.31	1.77
13	NEHUP 13	100	54.21	1.85
14	NEHUP 14	100	70.51	1.81

Sequencing and blast analysis

Sequencing of amplified product after PCR amplification using rbcL primer was performed using AB Big Dye technology. NCBI BLASTn (National Center for Biotechnology Information: Basic Local Alignment Search Tool) was performed with the DNA sequence data for identification and confirmation of species.

Results and Discussion

DNA Isolation

Molecular characterization of plants mainly depends on successful isolation of quality DNA as well as the use of a particular type of molecular markers. The quantity of isolated DNA also depends on the amount of grinded tissue harvested and collection of the supernatant (Moyo *et al.,* 2008).

The DNA isolation process is difficult to carry out in plant species containing secondary metabolites, such as in pumpkin seeds, which act as contaminants in the preparation of DNA leading to its degradation. Pumpkin seed contain higher levels of secondary metabolites such as polysaccharides, polyphenols, which may get co-precipitated with DNA and hinder the quality and yield of the isolated product. The removal of such compounds is essential to obtain good-quality DNA. Polysaccharide contaminations are particularly problematic (Scott and Playford,1996) and it can inhibit the activity of many commonly used molecular biological enzymes, such as polymerases (Fang *et al.,*1992), ligases and restriction end nucleases.

This is because nucleic acids form tight complexes with polysaccharides creating a gelatinous pellet and the embedded DNA is inaccessible to the enzymes (Sharma *et al.,* 2002). Polyphenol contamination of DNA makes it resistant to restriction enzymes as also shown in other taxa where polyphenol co purify with DNA (Katterman and Shattuck,1983) and interact irreversibly with proteins and nucleic acids (Loomis, 1974). DNA isolation protocols generally use CTAB to avoid co-purifying polysaccharides from plant tissues. Polyvinylpyrrolidone (PVP) has also been successfully used to remove polyphenols in CTAB DNA isolation protocols (Doyle and Doyle, 1990; Hills and Van Staden, 2002; Keb Llanes *et al.,* 2002; Schneerman *et al.,* 2002). Different percentages of PVP, 1, 2, and 4 were tried in the modified CTAB DNA isolation buffer as also proposed by others (Dellaporta *et al.,* 1983; 1%, Khanuja *et al.*,1999; 2%, Csaikl *et al.*,1998; 4%, Keb Llanes *et al.,* 2002) and it was found that 2% PVP gave optimum result. The quality of isolated DNA was checked by agarose gel electrophoresis (Fig. 1) and spectrophotometric readings. The yield of DNA as revealed by UV-VIS spectrophotometer quantification and reading absorbance at 260 nm and A280 has been reported and presented in Table 2.The results showed that the DNA isolated by this method gave a clear and sharp band in 1.5% agarose gel (Fig. 1). Total DNA isolated was also checked by means of agarose gel electrophoresis. The average DNA concentration obtained was 63.9µg/gm and the purity ranged between 1.66 to 1.85 (Table 3). The modified CTAB method yielded high-molecular weight pure form of DNA, free from secondary metabolites and other contaminants like protein, RNA etc from pumpkin seeds without using liquid nitrogen (Fig. 1).

Table 3. Primer sequences for species identification and diversity analysis.

Primer	Sequence (5' - 3')	Annealing Temp (°C)	Number of Bands		
			T: Total; P:Polymorphic; M: Monomorphic		
			T	P	M
rbcL	F: ATGTCACCACAAACAGAGACTAAAGC R: GTAAAATCAAGTCCACCRCG	54.5	1	0	1
RAPD (PPR1)	AATCGGGCTG	38.9	8	6	2
SSR (OCM1)	F: GGTCCCAATAATAGCAACCAA R: GTGGGACACATCTTGAGCA	52.8	1	1	0
SSR (OCM2)	F: GAAGTTCGTGGTCTGTGCAAGTC R: CCTGAGTAACCTCCGTGCTTCC	57.3	1	1	0

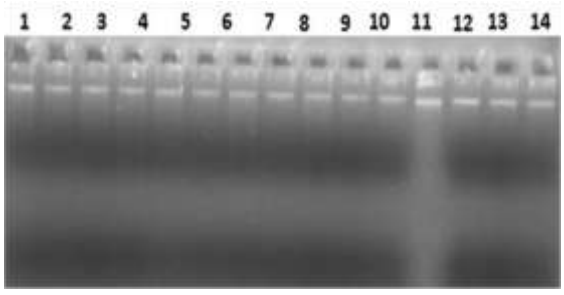

Fig. 1. Ethidium bromide-stained agarose gel showing total DNA isolated from fourteen samples of seed tissue of Pumpkin, *Cucurbita moschata* Duch. (lanes 1 to 14).

Primer analysis

The DNA isolated using the modified CTAB method from pumpkin seeds showed high intensity amplification with arbitrary RAPD and SSR primers (Fig. 2, 3 and 4) in PCR analysis. RAPD primer, PPR1 amplified bands for all 14 isolated pumpkin DNA samples and showed both monomorphic and polymorphic bands in gel electrophoresis (Fig. 2).

Isolated DNA samples were also amplified using SSR primers (OCM1 and OCM 2). OCM 1 primer showed amplification at the expected allele size of the primer that is at 273bp for only 7 isolated pumpkin DNA samples (lanes 2, 4, 6, 7, 8, 9 and 10) out of total 14 isolated pumpkin DNA samples (Fig. 3).

SSR primer, OCM 2showed amplification at the expected allele size of the primer that is at 300bp for 12 isolated pumpkin DNA samples (lanes 1, 2, 4, 6, 7, 8, 9, 10, 11, 12, 13 and 14) out of total 14 isolated pumpkin DNA samples. PCR amplification results using arbitrary RAPD and SSR primers showed high intensity amplification and good quality DNA bands in electrophoresis which can be used for diversity analysis.

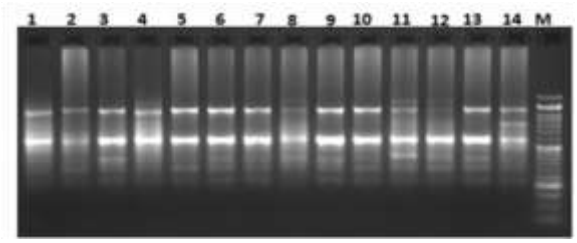

Fig. 2. Ethidium bromide-stained agarose gel showing PCR-amplified products obtained from DNA isolated from seed tissue by using random primer PPR1. Lanes 1-14: *Cucurbita moschata*, DNA and M: 50bp DNA ladder.

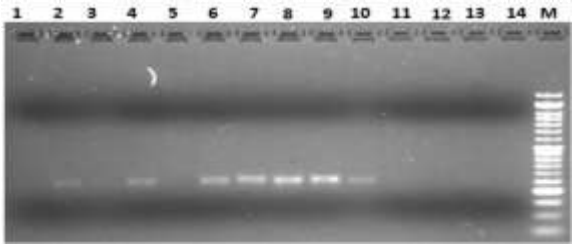

Fig. 3. Ethidium bromide-stained agarose gel showing PCR-amplified products obtained from DNA isolated from seed tissue by using SSR primer OCM 1. Lanes 1-14: *Cucurbita moschata*, DNA and M: 50bp DNA ladder.

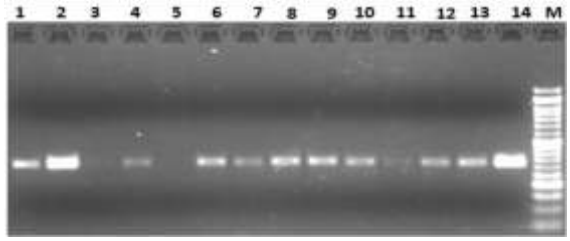

Fig. 4. Ethidium bromide-stained agarose gel showing PCR-amplified products obtained from DNA isolated from seed tissue by using SSR primer OCM 2. lanes 1-14:*Cucurbita moschata*, DNA and M: 50bp DNA ladder.

According to UPGMA cluster analysis, isolated 14 pumpkin seed DNA sampled were classified into two main broad clusters (Fig. 5). Broad cluster I comprised of only one genotype *viz.*

NEHUP8. Broad cluster-II was further subdivided into two major clusters with total 13 independent genotypes. The major cluster-I comprised of two genotypes. NEHUP 1 and NEHUP 14 with a genetic similarity percent of 0.58 approximately.

The major cluster-II was further sub divided in to three minor clusters. Minor cluster-I comprised of two genotypes viz. NEHUPc2 and NEHUP 4 with a genetic similarity percent of 0.64 approximately.

Minor cluster- II was again sub divided into three sub clusters. Sub-cluster I of minor cluster- II comprised of three genotypes viz. NEHUP 6, NEHUP 7 and NEHUP 9 with a genetic similarity percent of 0.80 approximately.

Sub-cluster II of minor cluster- II comprised of two genotypes viz. NEHUP 10 and NEHUP 13 with a genetic similarity percent of 0.85 approximately.

Sub-cluster III of minor cluster- II comprised of two genotypes *viz.* NEHUP11 and NEHUP 12 with a genetic similarity percent of 0.72 approximately. Minor cluster-III comprised of two genotypes *viz.* NEHUP3 and NEHUP5 with a genetic similarity percent of 0.60 approximately.

Blast analysis

PCR amplification of isolated pumpkin seed DNA sample using rbcL barcoding primers (codes for a product called "ribulose 1, 5-bisphosphate carboxylase/oxygenase; catalyzes the first step of carbon fixation) also produced DNA band in gel electrophoresis at the expected allele size of rbc L primers that is at approximately 700bp. Sequencing of amplified product has been done using AB Big Dye technology. NCBI BLASTn (National Center for Biotechnology Information: Basic Local Alignment Search Tool) analysis of sequence data also confirmed species identify for *Cucurbita moschata*.

The quantity and quality of the pumpkin seed DNA isolated by this modified CTAB method were high enough to perform more than 150 PCR reactions and also suitable for PCR reactions for species identification and diversity analyses.

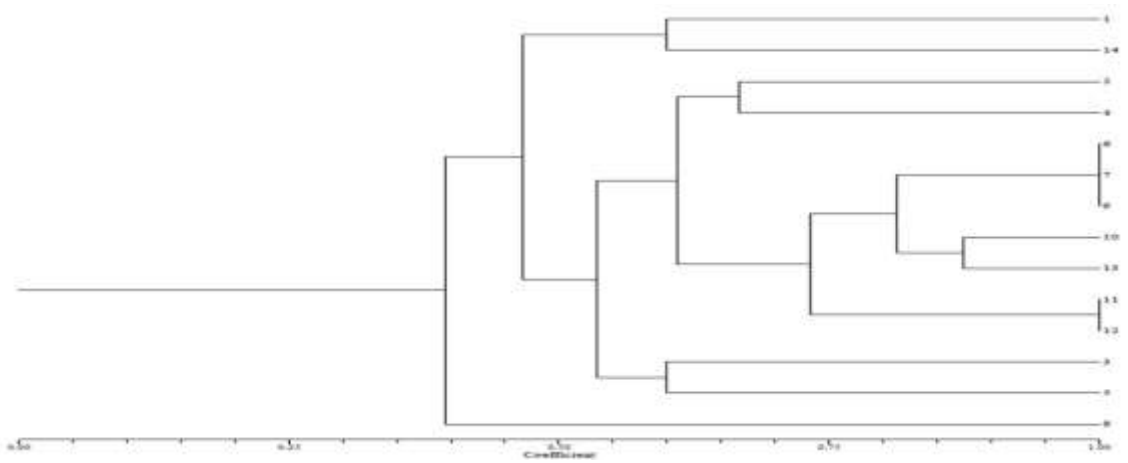

Fig. 5. UPGMA cluster analysis of isolated pumpkin seed DNA samples (1-14: *Cucurbita moschata* gentotypes).

Conclusion

The present study on development of modified method for isolation of high purity seed DNA without the use of liquid nitrogen and optimization of rbc L, RAPD and SSR primer conditions is a time saving method for species identification and diversity analysis as the modified method does not require seed germination and seedling development stages. The modified CTAB method resulted in high quality DNA yield which can be successfully utilized in PCR analysis. This will form a strong beginning for future molecular characterization and genetic improvement works in this promising cucurbitaceous plant.

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