A Simplified, Efficient and Rapid DNA Extraction Protocol from Rice Grains and Leaf

Abstracts

DNA extraction is very complicated with different plant species because the presence of secondary metabolites that hinder with DNA isolation processes and application like DNA restriction, Gene amplification, as well as gene cloning. A simple method for preparation of rice genomic DNA was developed. The current study expressed comparativelyrapid, cost-effective, less time consuming, methods for DNA extraction from seed, young leaves and old leaves of rice genotypes without using liquid nitrogen, phenol, and β–mercaptoethanol in extraction buffer. Using this methods, high quality and quantity of genomic DNA was get from 0.5 g of rice seed, young leaves and 75 days old leavesand extracted total nucleic acids (genomic DNA) was amplified by employing single sequence repeats (SSR) or microsatellite markers, which produced reproducible results.This protocol resolves the problems of DNA degradation, contamination, and low yield due to binding and/or coprecipitation with starches and polysaccharides.

Keywords:DNA isolation; PCR; *Oryza sativa;* Molecular Marker, DNA extraction and Molecular Breeding

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Introduction

The word “Dhan” is developed from Sanskrit word “Dhanya” which meaning “Sustainer” of the human race. Rice (*Oryza sativa*) belongs to the family Poaceae and sub-family *Oryzoideae*.Rice is a staple food that feeds over 50% population of the world; thus, it is essential in global food security, which contains approximately 80% carbohydrates, 7-8% protein, 3 % fats and 3 % fibers [1]. Rice is most important agronomic, monocot model crop, grown under the different eco-geographical environment in various tropical and sub-tropical countries. The genome size of rice is 430 Mb. Breeding techniques viz. Hybrid rice technology, molecular breeding, Quantative Trait Loci (QTL) analysis etc. used for rice have attracted worldwide concern from both the public and private sectors. Molecular marker-assisted selection (MAS), have been broadly applied in rice in previous studies [2].

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Molecular marker-assisted selection (MAS), quantative trait loci (QTL) analysis, evaluation of genetic diversity, conformation of transgenic and germplasm identification are important techniques in modern breeding programs. Hundred to thousand samples required to be practice for the MAS, QTL, screening of transformants as well as molecular studies for requiredrapid DNA isolation methods for such kind of protocols. Several authors [3, 4, 5, 6,7]explain DNA extraction protocols are mostly used in plant biotechnology and/or plant molecular biology, but available different methods comparatively expensive, used hazardous chemicals, time consuming, involved multiple steps, required expensive enzymes and used liquid nitrogen for grinding the samples [5, 7, 8]. The Cetyl-trimethyl-ammonium bromide (CTAB)is frequently used surfactants in DNA isolation include the lyses of cell wall as well as cell membrane, which release the genetic material into extraction buffer. CTAB protocols arevery famous methods for rice DNA extraction, including other plants, organism such as bacteria [9], Fungi [10], nematods [11], and animals [12]. Several modifications have been present in CTAB protocols [6,13, 14, 15]. Some protocols have been informed to reduce the DNA isolation steps but required more amount of plant tissue samples and liquid nitrogen[16]. The modified CTAB isolation protocols employing high concentration of salts to used removed polysaccharides because huge quantity of polysaccharide in samples the problematic at the time of PCR reaction.

A number of protocols are available and are being developed for extraction of DNA from plants. While, diverse plants contain different amounts of DNA, Isolation of high-quality nucleic acids is a prerequisite for MAS studies. Many DNA extraction protocols have been developed for rice [17]. However, these protocols typically involve the use of living organs such as green leavesas experimental materials; requiring researchers to germinate the seeds and to select the target plants at the seedling stage based on polymerase chain reaction results. It needs few weeks or few months from plantation to fresh tissuecollection and also requires more attention for management practices. To overcome these problems to initiatenon-destructive detection methods by isolating nucleic acids from triploid embryos [18],developed a nucleic acids isolation protocols using the dry half seeds of rice[5, 14],develop a rapid protocol for DNA extraction from seed of rice.

However, in the protocols provided by [17, 18, 19], the concentrations of nucleic acids from cereal crops (rice, wheat and maize) were relatively low. Most laboratories wish a simple and fast procedure for obtaining plant genomic DNA for PCR, and good-quality DNA for complete enzyme digestion. Therefore, a protocol for extracting genomic DNA from young or

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old, fresh or dry rice leaves, dry seeds, panicle, and stems that is applicable to a variety of plants, regardless of the complexity of their genomes is presented. The objective of this study was to develop a simple and rapid method to isolate DNA under normal laboratory condition (room temperature) from small amount of tissue for large number of samples.

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Material and Methods

Materials

In present study we were used the Basmati rice and non-Basmati plant material of three stage viz. dry seeds, 15 days old leaf (grown in small Aluminum pots) and 65 to 70 days old leaf (grown in field condition) for isolation of DNA. All leaves sample were collected from 15 days old plant raised in pots and 70 days old plants grow in field, wrapped in aluminum foil and kept immediately in an ice containing box.

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Primers

Isolated nucleic acids were amplified with Simple Sequence repeat (SSR) markers and specific primers (Table:1).

Table 1. List of twenty four primers utilized for amplification of rice genomic DNA extracted from twelve basmati and non basmati entries used in the study.

S.N.	Primers	Chro No.	Forward	Reverse	Motif Repets
1.	RM 1024	5	GCATATACCATGGGGATTGG	GGGATTGGGATAATGGTGTG	(AC)13
2.	RM 1183	1	GGGCACGAATAAAACCAGAG	GGGATGGTCCAATGACAAAG	(AG)14
3.	RM 1018	4	ATCTTGTCCCACTGCACCAC	TGTGACTGCTTTTCTGTCGC	(AC)13
4.	RM 3333	4	AAGCTATCGACACCGTGACC	GCACCTTACAATTTGGCACC	(CT)15

Reagents

The solutions used were: 1M Tris-HCl (prepared using 3.94 gTris-HCl dissolved in 20 ml deionized water & pH8.0 adjusted using 1N HCl and/or 5M NaOH solution, makeup the 30ml volume with double distilled water), and 0.5M EDTA (pH 8.0) (prepared using 4.65 g ofEDTA dissolved in 15 ml de-ionized water and 25 ml final volumemakeup with de-ionoized water). The 5MNaOH and 1N HCl solution was used to adjust the pH to 8.0. The 2.0M NaCl(11.58 g NaCl added into 40 ml of de-ionized water and adjudgedthe final volume to 100 mL with de-ionized water); 3% CTAB (3 g CTABdissolved into 100 ml de-ionized water); and 70% ethanol (75 ml absolute ethanol mixed with 25 ml de-ionized water.The 3X extraction buffers was prepared by using 3 % (w/v) CTAB solutions 2 % PVP,1MTris-HCl (pH8.0), 0.5M EDTA (pH 8.0), and 2M NaCl (Table-2). The another solution chloroform: isoamyl alcohol (24:1) (CI)(Table 3), 50X

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Tris-acetate-EDTA (TAE) buffer (Table 4), and 1X TE buffer (Table 5) theircomposition and preparation are available.

DNA extraction procedure

The young leaves were collected from 15 days old seedling raised in the pots, wrapped in aluminium foil and kept immediately in a box containing ice. Same procedure used in 70 days old field growing rice plant and taking seed are stored in laboratory. 0.5 to 1.0 gram fresh young and older leaf sample and 10 to 15 grain were grind in mortar and pastel in 2000 µl 3X extraction buffer.

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Table 2: Composition (only for 100ml) and Prepration of 3X CTAB extraction Buffer solution.

Reagent	Weight in gram	Stock Solution	Final Concentration	100ml prepration
Tris-HCl	3.94	1.0 M	100 mM	10 ml
EDTA	4.65	0.5 M	20 mM	10 ml
NaCl	11.58	4.95 M	2.0 M	40 ml
CTAB	3 (w/v)	---	3 %	3 g
PVP	2 (w/v)	---	2 %	2 g
Double Distilled Water		---	---	30 ml*
Final Volume =				100

* Water amount more or less as required to maintain 100 ml final volume.

Table 3: Composition and prepration of fresh Chloroform: Isoamyl alcohol (24 :1) without phenol

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S.N.	Reagents	100 ml preparation
1.	Chloroform	96.0 ml
2.	Isoamyl alcohol	4.0 ml
Total		100.0 ml

Table 4: Composition and prepration of the 50X and 1X TAE Buffer

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S.N.	Reagent	1 Litre 50X Buffer	1 litre 1 X TAE Buffer
1.	Tris Base (pH8.0)	242.4 g	20.0 ml TAE + 980.0 ml DD H2O
2.	Glacial Acetic Acid	57.3Ml	
3.	0.5M EDTA (pH 8.0)	100 ml	

Table 5: Composition and Prepration of the 1X TE Buffer

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S. N.	Reagents	Stock Solution (M)	Final Concentration (mM)	250 ml prepration
1.	Tris-HCl (pH 8.0)	1.0	10 mM	2.5 ml
2.	EDTA (pH 8.0)	0.5	1.0	0.5 ml
3.	dd H2O	----	---	247 ml

The homogenised mixture transfer in 2.0 ml eppendorf centrifuged tube and the content were mixed gently by swirling and inverting the tube and incubated at 64 ˚C in water bath for 50 min to 1 hour with occasional mixing at 10 to 15 min interval. The tube were taken out and equal volume of Chloroform: isoamylalcohol (24:1) was added. The content were mixed by inversion for 10 min and centrifuged at 10,000 rpm for 10 min. The clear aqueous layer was transferred to new tubes and re-extracted with an equal volume of CI and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred into new tubes. Two third volume of isopropanol was added and mixed gently by inverting the Eppendorf tube and placed at -20 ˚C for 30 min (4 ˚C for the whole night). Genomic DNA was pelleted by centrifuging at 10,000 rpm for 10 min and the pellet was wash with 300 µl Ethanol (70%) and centrifuging at 10,000 rpm for 5 min. The DNA pelleted was air dried and dissolved in 30 µl TE buffer by gentle tapping and isolated genomic DNA was stored at -20 ˚C for further use.

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Quality Determination of isolated DNA

The determination of quality of isolated DNA is essential to assess the suitability of good quality DNA for PCR amplification. The isolated genomic DNA samples were subjected to electrophoresis through agarose gel to assess the quality of DNA isolated from the seed, leaves of young seedlings, and 70 days old rice plant achieve from the genotypes under evaluation in the present study. The DNA sample 3 µl were mixed with 1/8th volume of 6 X DNA gel loading dye and was loaded onto 0.8 % agarose gel containing of ethidium bromide, in a submarine horizontal electrophoresis unit filled with 1X TAE buffer. Electrophoresis was carried out at 90V for 45 min. The gel was viewed under UV light to detect the quality of DNA. The DNA fragments were visualized as fluorescent bands because of fluorescence of ethidium bromide intercalated between the nitrogenous bases of DNA under UV light. Appearance of a single, sharp band of high molecular weight without smearingindicated the extraction of a good quality DNA sample with uniform segment size andless damage during extraction process.

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Amplification of genomic DNA and separation amplified DNA through agarose gel electrophoresis

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The primer vials were centrifuged before and after the addition of 1X TE buffer to the vials. After dilution the concentration of each primer stock solution was obtain as 100 µM. The diluted primers were stored at -20^{0}C.Amplification of DNA was carried out using standard protocol of polymerase chain reaction (PCR) adjusted to laboratory conditions. The amplification was carried out in a thermal cycler using 15 µl of reaction mixture (Table 6). The reaction mixture was prepared by varying the components involved in composition of reaction mixture.

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Table6: Composition of PCR reaction mixture (15 µl)

Sl. No.	Components	Volume (µl)
1	Water (Protese and Nuclease free)	3.0
2	5X PCR Buffer	2.8
3	$MgCl_2$ (10 mM)	1.4
4	dNTPs (200 µM)	2.8
5	Primer F (5 µM)	1.0
6	Primer R (5 µM)	1.0
7	Taq DNA polymerase (1 unit)	1.0
8	Template DNA (Diluted1 : 9 (DNA:H2O))	2.0
	Total	15.0 µl

The polymerase chain reaction was performed with the help of microsatellite sites based 3 pairs of forward and reverse simple sequence repeat (SSR) primers. The amplification was carried out in a thermo-cycler using the program standardized for this purpose. The amplified product was separated using 2.0 % agarose gel with ethidium bromide as staining reagent. The amplified product with expected product size above 100 bp was subjected to 2% gel electrophoresis at 90 V for one hour and then visualized and documented in gel documentation system. The size of the amplified product was estimated with the help of a 100 bp ladder.

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Result and Discussion

The superior quality of DNA sample was obtained from seed, 15 days young leaves, and 70 days old leaves, of twelve rice (*Oryza sativa*) genotypesand amplified using four simple sequence repeat (SSR) or microsatellite markers namely viz. RM 1024, RM 1183, RM 1018, and RM 3333. The amplified product was observed in all samples tested with little variation in intensity of the amplified bands. After amplification RM 1024 produced 110-142 bp, RM 1183produced 113-142 bp, RM 1018 produced 135-149 bp, and RM 3333 produced 160-189 bp polymorphic productand allelic size difference 32, 29, 14 and 29 bp respectively (Figure 1 and 2). Here is described a simple and reproducible procedurefor PCR amplification of rice DNA, that also is applicablefor other plant genomes.

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DNA samples prepared using the extractionprocedures (lane1 = Marker (M), 2-5 Seed genomic DNA, 6-9 young leaves Genomic DNA and 10-13 mature 70 days old genomic DNA in Figure 1) were subjectedto PCR amplification using different primers: RM 1024 onchromosome 5, RM 1183 on chromosome 1, RM 1018 on chromosome 4, and RM 3333 on chromosome 4, and the repeat motif of SSRmarkers is $(AC)_{13}$ $(AG)_{14}$, $(AC)_{13}$ and $(CT)_{15}$ respectively. All the genomic DNA samples produced aclear, sharp, and reproducible PCR product when theprimers were used for PCR amplification (Figure 2).Although two variations of the DNA extraction procedurewere used, there was no difference between lanes of twoprotocols.

Numerous protocol for plant genomic DNA extraction are available however, the better results were obtained with those utilized CTAB [1, 5, 7, 16, 19, 20].In this experiment is anxious withmodification of CTAB method to isolated DNA without using liquid nitrogen, β-mercaptoethanol, andphenol.The concentration of NaCl washigh(2M) which extracted high quantity ofDNA.The main action of NaCl in extractionbuffer is the removal of proteins andcarbohydrates which are attached to the DNA. Polyvinylpyrrolidon form H-Bond with phenolic metabolites and prevent the plant genomic DNA contamination by phenolic metabolites [13. 16, 17,18]. There are several advantage in using genomic DNA extraction protocol issuitable for polymerase chain reaction (PCR)genotyping, which can be employed for therecognition of alleles in diverse genetic,breeding and biotechnological approaches, viz. markerassisted selection (MAS), quantative trait loci (QTL), genetic mapping,and Transgenic Screening, mutant introgression. All four microsatellite markers show very good amplification and polymorphism with the DNA extracted through this method. Therefore, this method should be recognizing as a good rapid and inexpensive method for DNA extraction from rice leaves and seeds.

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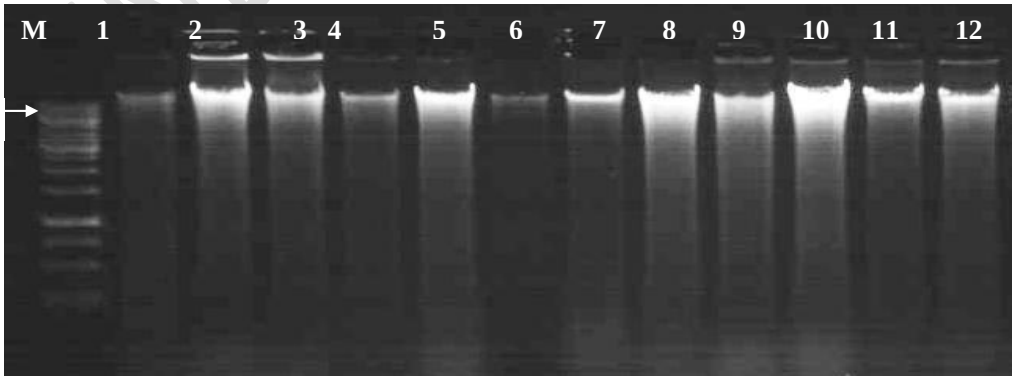
Comment [l41]: advantages



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M-Marker	**1. Nagina-22**	**2. Punjab Basmati-4**	**3. Kasturi**	**4. Pusa Basmati-1121**	**5. Ranvir Basmati**	**6. CSR-30**
	7.Vallabh Basmati-23	**8. IR-64**	**9. Pant Basmati-1**	**10. Sarbati**	**11. Vallabh Basmati 24**	**12. Pusa Basmati -1**

Figure 1: Crude total genomic DNA isolated from twelve different varieties1 to 4 isolated from seeds, 5 to 8 isolated young leaves and 9 to 12 isolated 70 days older rice genotypes.

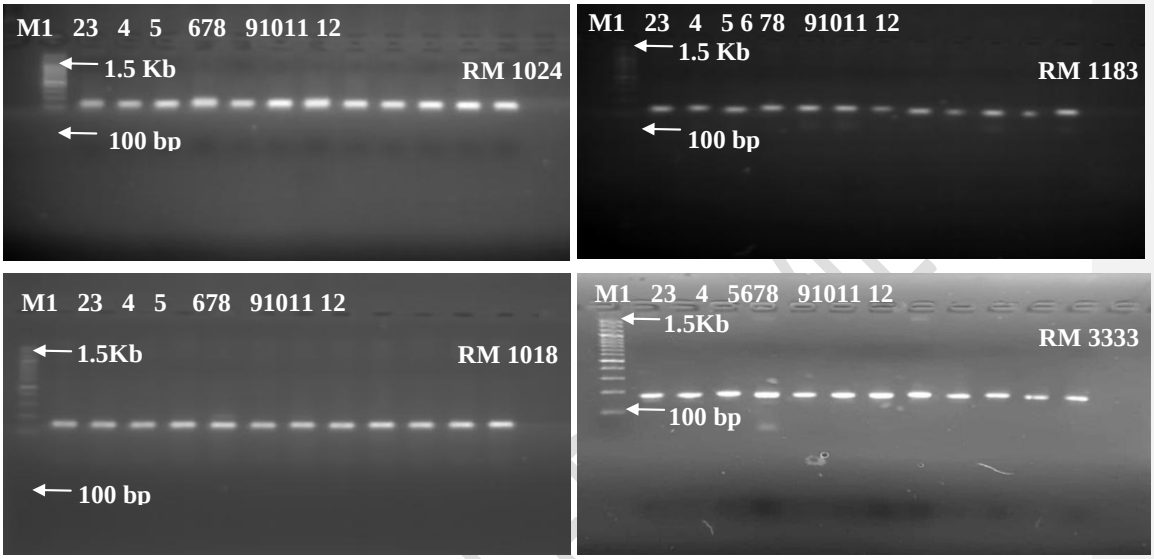

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Figure 2: Amplification of genomic DNA with selected four different primers with all genotypes.

Conclusions

In summary, the procedure for rice genomic DNA or nucleic acids extraction described here could be a trusted and reliable protocol to work well for analysis without using liquid nitrogen, 2-mercaptoethanol, and Phenols.

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