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Evaluation of DNA Extraction Methods for RAPD, SSR and AFLP Analyses of Wild Rose Species

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ABSTRACT

The current study aims to evaluate a rapid, simple and robust method of DNA extraction for AFLP analysis of 12 wild rose species (*Rosa brunonii*, *R. cathayensis*, *R. moschata*, *R. multiflora*, *R. wichurriana*, *R. indica*, *R. alba*, *R. macrophylla*, *R. tomentosa*, *R. canina*, *R. damascena*, *R. bourboniana* and the F1 progeny of *R. damascena* and *R. bourboniana*). Extraction of quality DNA from wild rose species is difficult as they contain high levels of polysaccharides and polyphenols. Four DNA extraction protocols were compared: two commercial kits from Qiagen and AuPrep, CTAB and a modified CTAB protocol. The protocols were evaluated in terms of yield, purity, restrictability and amplifiability of recovered DNA. The yield and quality of genomic DNA was considerably affected when commercial kits and common CTAB protocol were utilized for DNA isolation. The modified phenol free, CTAB procedure involving a washing step before extraction was the most successful extraction method giving optimum yields (900-1750 µg/g) of quality DNA that was amenable to restriction digestion and polymerase chain reaction (PCR) analyses – RAPD, SSR, AFLP.

Keywords: polysaccharides, polyphenols, RAPD, SSR

INTRODUCTION

Understanding natural variation within ornamental and wild relatives of rose is necessary for overall improvement and successful management of the crop. Wild roses are a source of potential genes for cultivating new species. Incorporation of these genes into economically important varieties can yield superior cultivars. Roses are a source of fragrant essential oil which is used in making high-grade perfumes. It is also used in medicine as it can regulate *qi* and invigorates the blood and has an effect of astringency (Chrubasik *et al*. 2006; Fu *et al*. 2006). The fruits can be used in food and medicine; the big and beautiful flowers of rose have great ornamental and economic value. Significant progress made in molecular approaches has gained increasing popularity of PCR based molecular markers, over morphological, protein and isozyme markers (Geuna *et al*. 2000). Today, they are commonly employed in fingerprinting of genotypes, molecular mapping, taxonomic affinities, evaluation of biodiversity and marker assisted selection in vegetatively propagated crops. All these techniques require simple, rapid, and reliable protocols for extracting quality DNA. Unfortunately, roses contain high levels of polysaccharides, polyphenols, and other secondary metabolites (Wen and Deng 2002), which render DNA unusable for downstream applications (Pirttila *et al*. 2001). Polysaccharides make DNA viscous, glue-like and reduce *Taq* enzyme activity in PCR reaction (Porebski *et al*. 1997). Polyphenols bind covalently with DNA, make DNA brownish, indigestible, and poorly yielding. Obtaining good-quality DNA is a key obstacle for molecular studies of rose. A number of methods for DNA isolation have been devised from time to time for various rose species (**Table 1**). DNA isolated by common CTAB, SDS and a few modifications thereof, is sufficiently pure for routine PCR amplification – RAPD, AP PCR, microsatellite and RFLP analysis. But for more demanding applications were DNA serves as a substrate for restriction endonucleases and ligases (e.g. AFLP), Qiagen's DNeasy Plant mini kit and modified CTAB methods by Suhl and Korban (1996), Kobayashi *et al*. (1998), and Zang *et al*. (2001) have been utilized for obtaining quality DNA in rose species (**Table 1**). But these protocols are either long, tedious or costly. Methods developed for other plants (Aljanabi and Martínez 1997; Peterson *et al*. 1997; Porebski *et al*. 1997; Cheng *et al*. 2003), containing high levels of polyphenols and polysaccharides have failed to isolate pure, restrictable DNA from rose species. In this context, the present study aims to evaluate four DNA isolation methods based on DNA yield, restriction digestion and amplification quality, for development of molecular markers to assess genetic diversity within the germplasm and also for evaluating transgenic lines.

MATERIALS AND METHODS

Plant material

Plant material consisted of 85 rose accessions belonging to 12 wild rose species – *Rosa brunonii*, *R. cathayensis*, *R. moschata*, *R. multiflora*, *R. wichurriana*, *R. indica*, *R. alba*, *R. macrophylla*, *R. tomentosa*, *R. canina*, *R. damascena*, *R. bourboniana* and the F1 progeny of *R. damascena* and *R. bourboniana*.

DNA extraction

Total genomic DNA was extracted from fresh leaf tissue (1.0-2.0 g) fixed in liquid nitrogen and stored at -80°C until extraction by the following four methods. Initially, for comparison, DNA was isolated from 46 accessions representing four rose species (see **Appendix 1**) and subsequently the best developed protocol was utilized for DNA isolation from all the accessions.

1. DNeasy Plant Mini Kit

Protocol
Grind 100 mg of leaf tissue to a fine powder in liquid nitrogen using a mortar and pestle. Transfer powdered material to pre warmed AP1 buffer (400 µl) in an Eppendorf tube followed by other steps as per the manufacturer's (Qiagen) instructions.

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Techniques Paper

Table 1 DNA extraction protocols utilized for *Rosa*.

Protocol	*Rosa* group, species or section	Purpose of DNA analysis	Difference from present protocol
SDS-based (Martin *et al*. 2001)	Groups: albas, bourbons, damascenas, centifolias, gallicas, multifloras, noisettes, perpetuals, portlands, semipervirens, teas and hybrid teas	AP PCR	Lysis with 1% SDS. Deproteination with phenol chloroform.
CTAB (modified by Saghai-Maroof *et al*. 1986)	*R. floribunda*, *R. clinophylla*, *R. moschata* (Sasikumar *et al*. 2007)	RAPD	2-h extraction with extraction buffer. Use of chloroform: octanol (24: 1) for purification. RNase treatment for 4 h.
CTAB (Doyle and Doyle 1987)	*R. gymnocarpa*, *R. pisocarpa*, *R. multiflora* (Joly *et al*. 2006); *R. canina*, *R. dumalis* (Aras 2003)	PCR amplification of leafy gene, RAPD	Treatment with RNase given prior to incubation (at 65°C) with extraction buffer. Chloroform isoamyl alcohol extractions carried out twice.
Modified CTAB method (Vosman *et al*. 1992)	*R. hybrida*, *R. canina* L., *R. indica* Thory., *R. chinensis* Jacq., *R. rubiginosa* L., and *R. rubrifolia glauca* Pour. (Esselink *et al*. 2003); *R. multiflora* (Yan *et al*. 2005)	AFLP, SSR, SCAR, RFLP	Isolation of nuclei prior to lysis. DNA purification carried out by phenol/chloroform extraction.
Modified CTAB (Suhl and Korban 1996)	*R. damascena*, *R. multiflora*, *R. persica* (Debener *et al*. 1999; Basaki *et al*. 2009)	AFLP, RAPD	Extraction carried out with 8M LiCl in extraction buffer. Samples extracted 3 times with chloroform. Digestion of carbohydrates in resuspended DNA samples carried out with drislase at room temperature. DNA purification with phenol chloroform.
Walker and Werner (1997)	*R. moschata R. chinensis* hybrids (Frederick *et al*. 2002; Wagner *et al*. 2002)	RAPD analysis	Lysis buffer contains sodium acetate and 1.5% SDS.
Modified CTAB (Kobayashi *et al*. 1998)	*R. rugosa*, *R. roxburghii*, *R. multiflora* (Hattendorf *et al*. 2007)	AFLP, SCAR	Purification of DNA carried out with phenol: chloroform.
Modified CTAB (Lefort and Douglas 1999)	*R. damascena* Mill. (Rusanov *et al*. 2005)	Microsatellite analysis	Extraction buffer contains LiCl (0.4 M), CTAB (1%), PVP(1%). DNA yield 20 ng/µl.
Modified CTAB (Zhang *et al*. 2001)	*R. hybrida* x *R. wichuriana* interspecific hybrids, *R. chinensis* (Crespel *et al*. 2002)	AFLP; positional cloning	Nuclei extraction buffer includes sucrose, trizma, spermide, spermine and triton X 100 whereas lysis buffer contains sarcosine and proteinase K.
Modified CTAB (Cheng *et al*. 2003)	*R. roxiburgii*	PCR and RFLP analysis	Water saturated ether used for DNA purification. DNA yield ranged from 50-500 µg/g.
Modified CTAB (Joly *et al*. 2006)	*R. blanda*, *R woodsii*, *R. foliolosa*, *R. nitida*, and *R. palustris*	gene amplification	Modifications involved scaling the protocol for a total CTAB volume of 600µl; adding 12 µl of 0.5 mM ethylenediamine tetra-acetic acid (EDTA) pH 8.0 per 600 µl of CTAB and 1% polyvinylpyrrolidone (PVP) to the extraction buffer prior to extraction; adding 20 µl of RNAse A to the CTAB buffer prior to incubation at 65°C; performing two chloroform-isoamyl alcohol (24: 1) extractions and precipitating the DNA with 1.5 volumes of 100% ethanol.
DNeasy Plant Mini Kit (Qiagen)	*R. damascena*, *Rosa* section Caninae, Tomentellae, Vestitae, Rubigineae, Rubrifiliae, Syntilae (Baydar *et al*. 2004; Nybom *et al*. 2006; Babaei *et al*. 2007; De Cock *et al*. 2008)	SSR, STMS AFLP, SCAR	Uses silica gel membrane technology based spin columns.
Modification of DNeasy Plant Mini Kit (Qiagen, Inc.) Aegerter *et al*. (2002)	Rose rootstock	RAPD	Replacement of supplied lysis buffer with 2.5% CTAB, 1% polyvinyl pyrrolidone (PVP-40), 1.4 M NaCl, 50 mM EDTA, and 100 mM Tris-HCl (pH 8.0). Replacement of supplied elution buffer with 10 mM Tris-HCl at pH 9.0. The elution step was conducted twice with 100 µl of buffer each time for a final elution volume of 200 µl.

2. Au Prep™ DNA easy plant mini Kit extraction

Protocol

Grind 100 mg leaf sample under liquid nitrogen and mix with 400 µl of buffer PX1 and 4 µl RNase stock solution followed by other steps as per the manufacturer's (Life Technologies, India) instructions.

3. CTAB extraction

Chemicals and reagents

All chemicals used in the method were purchased from Sigma. Reagents required are:

- CTAB isolation buffer (2% hexadecyltrimethylammonium bromide (CTAB), 2% polyvinylpyrrolidone (PVP – MW 40000), 1.4 M NaCl, 0.2% β-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0)
- Chloroform-isoamyl alcohol (24:1 v/v)
- 3 M potassium acetate (pH 4.8)
- Isopropanol
- TE buffer (pH 8.0): 10 mM Tris-HCl, 1 mM EDTA
- 10 mg/mL DNase-free RNase A
- Absolute ethanol
- 70% ethanol
- Distilled autoclaved water

Protocol

Preheat CTAB solution to 65°C. Grind 1-2 g of leaf tissue into a fine powder in liquid nitrogen using a mortar and pestle. Add the powder to pre warmed isolation buffer in an oak ridge tube. Vortex thoroughly and place the tube at 65°C. Hold the tube at 65°C for 60 min and vortex 3-4 times during the incubation. Add equal volume of chloroform- isoamyl alcohol (24: 1) and mix thoroughly, followed by other steps as per the protocol of Doyle and Doyle (1987, 1990).

4. Modified CTAB extraction

Chemicals and reagents
All chemicals used in the method were purchased from Sigma. Reagents required are:
• Washing buffer (100 mM Tris-HCl (pH 8.0), 5 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 0.35 M glucose, 2% polyvinylpyrrolidone (PVP MW 40000), 4% β-mercaptoethanol)
• Extraction buffer (100 mM Tris-HCl (pH 8.0), 1.5 M NaCl, 50 mM EDTA (pH 8.0), 4% β-mercaptoethanol, 3% (w/v) CTAB)
• Chloroform-isoamylalcohol (24:1 v/v)
• 5 M potassium acetate (pH 5.8)
• 3 M sodium acetate
• TE buffer (pH 8.0): 10 mM Tris-HCl, 1 mM EDTA
• 10 mg/mL DNase-free RNase A
• Isopropanol
• 70% ethanol

Protocol
Grind 2 g of leaf tissue in liquid nitrogen with a mortar and pestle. Transfer the ground powder to a clean, autoclaved 50-ml Oakridge tube and add 20 mL of washing buffer (100 mM Tris-HCl (pH 8.0), 5 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 0.35 M glucose, 2% polyvinylpyrrolidone (PVP), 4% β-mercaptoethanol). Vortex the samples thoroughly and incubate on ice for 30 min. This is followed by other steps as per the protocol of Xu *et al*. (2004) except that the chloroform isoamyl alcohol extraction is done only once and the pH of 5 M potassium acetate is maintained at 5.8.

Comparison of efficiency of the extraction protocols

DNA quality and quantity was verified with a spectrophotometer and electrophoresis using 0.8% agarose gel and uncut λ DNA (New England Biolabs, USA) as a standard. The success of the extraction method was evaluated by the DNA concentration, spectral absorbance ratios at $A_{260}/_{280}$.

RAPD analysis

PCR was performed in a 25 µl volume of reaction mixture (Williams *et al*. 1990) containing 1 X *Taq* Polymerase buffer (with 25 mM $MgCl_2$), 0.6 units of *Taq* DNA Polymerase (Bangalore Genei, India), 5 mM dNTPs (MBI Fermentas), 10 mM of random decamer primer (Finnzymes) and 15 ng of total genomic DNA. Amplifications were carried out using a DNA thermo cycler (Bio-Rad Laboratories, USA) with the following parameters: 1 cycle of 4 min at 94°C followed by 45 cycles of 1 min at 94°C, 1 min at 37°C and 2 min at 72°C. The last extension cycle was programmed at 72°C for 7 min. The PCR products were resolved on a 1.5% agarose gel containing 0.4 mg/l ethidium bromide and photographed using a gel documentation system.

SSR analysis

DNA amplification was carried out in a 10 µl reaction volume according to Yan *et al*. (2005) containing 1X PCR assay buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 25 mM of each dNTP, 20 ng of each forward and reverse primers, 0.2 U of *Taq* DNA polymerase (Bangalore Genei) and 20 ng of DNA. The amplification reaction was carried out as above in a thermo cycler programmed at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min and a final extension at 72°C for 7 min. PCR products were separated on a 3% metaphor agarose gel using 1X TBE buffer. The size of amplified fragments was determined by using 100 base pair DNA ladder (MBI Fermentas, Lithuania) as size standard. The SSR profiles were visualized and captured using the Alpha Digi Doc Gel Documentation and Image Analysis System (Alpha InfoTech, USA).

AFLP analysis

Genomic DNA (250 ng) was digested to completion with *Eco*RI + *Mse*I in a total volume of 25 µl (Vos *et al*. 1995) by incubating at 37°C for 2 hrs. After heat inactivation at 70°C for 15 min, *Eco*RI- and *Mse*I-specific adapters were ligated to the digested DNA fragments. The adapter ligated DNA was preamplified using *Eco*RI and *Mse*I pre-amplification oligos with one selective nucleotide. The pre-amplified library was diluted with sterile water in a ratio of 1: 50. Selective amplification was carried out using γ-P33-ATP labelled *Eco*RI oligo in combination with *Mse*I oligo, each with 3 selective nucleotides at the 3′ end. An equal volume of formamide dye was added to the amplified products and electrophoresed on 6% PAGE under denaturing conditions and then autoradiographed (Sambrook *et al*. 2001).

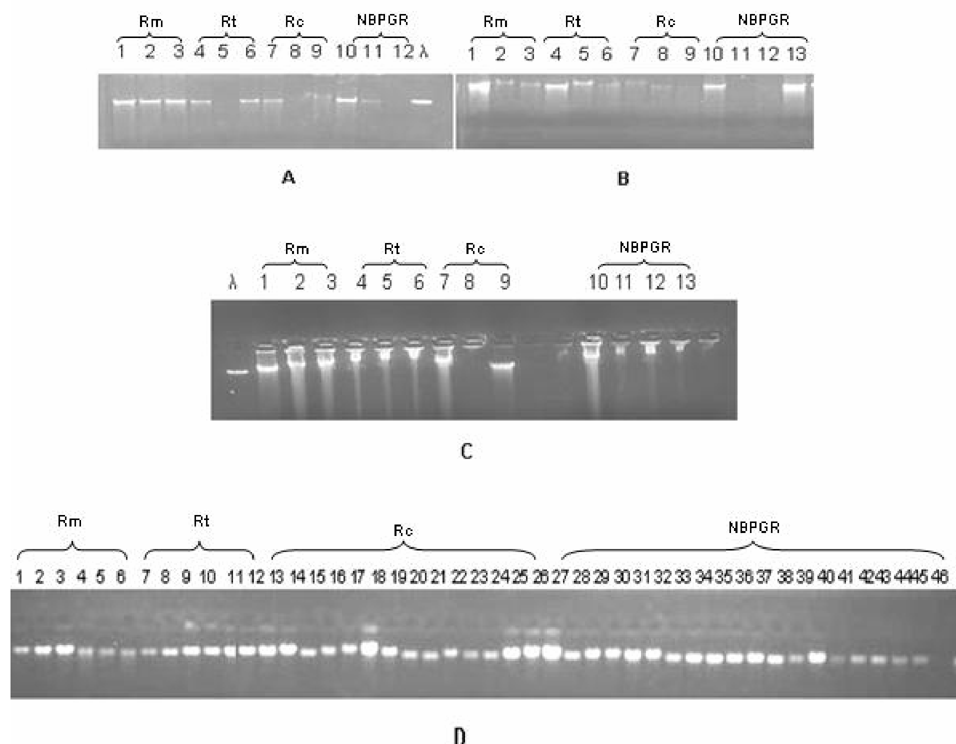

Fig. 1 DNA isolated from wild rose accessions using methods 1 (**A**), 2 (**B**), 3 (**C**) and 4 (**D**). Lanes 1-12/13 (ABC) and 1-46 (D) represent DNA samples of *Rosa macrophylla* (Rm), *Rosa tomentosa* (Rt), *Rosa canina* (Rc) and accessions from NBPGR, Shimla (NBPGR). λ = uncut lambda DNA (50 ng).

RESULTS AND DISCUSSION

Yield and quality of genomic DNA was considerably affected by the isolation methodologies utilized for DNA extraction from the rose accessions (**Fig. 1**). Extraction methods 1 and 2 produced extremely little DNA yields. Protocol 1 gave higher DNA yield than protocol 2. The ratio of absorbance $A_{260}/_{280}$ nm ranged from 1.6-1.8; however DNA yields were not same in all the accessions of wild rose (**Fig. 1A, 1B**). Method 3 (**Fig. 1C**) resulted in co-precipitation of polysaccharides and DNA. DNA obtained in most of the samples was dirty yellow in appearance with high viscosity. DNA isolation protocols generally use CTAB to avoid copurifying polysaccharides from plant tissues. Keeping this in mind we tried several modifications, including an increase in the concentration of CTAB to 3% (tried by Khanuja *et al*. 1999, for diverse medicinal and aromatic plants), β-mercaptoethanol to 1% (Tel-zur *et al*. 1999, for epiphytic cacti), and NaCl to 2.2 M (Aljanabi *et al*. 1999, for sugarcane) and also different percentages of PVP (1 and 2%) as also proposed by others (1%, Khanuja *et al*. 1999, for diverse medicinal and aromatic plants; 2%, Dellaporta *et al*. 1993, for maize and *Nicotiana* and Csaikl *et al*. 1998 for oak, elm and pine). These modifications were tried either alone or in combinations but the DNA yield remained unsatisfactory in terms of quality for endonuclease-based marker analysis.

Method 4 produced optimal yields (900-1750 μg/g) of quality DNA (**Fig. 1D**). The DNA produced was amenable to restriction digestion with *Eco*RI/*Mse*I endonucleases (**Fig. 3A**) and successful AFLP fingerprinting with different primer combinations (**Fig. 3B**). The ratio of absorbance $A_{260/230}$

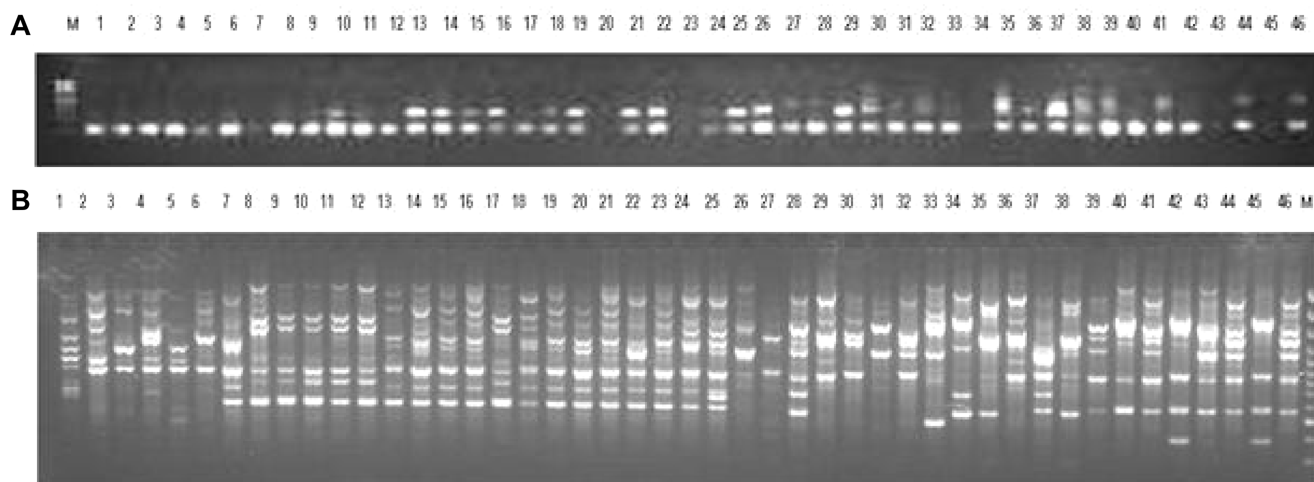

Fig. 2 (**A**) SSR profile of 46 wild rose accessions amplified with primers of RhB510 locus. (**B**) RAPD profile of 46 wild rose accessions amplified with OPV12. Lanes 1-6 represent accessions of *Rosa macrophylla*; lanes 7-12 represent accessions of *Rosa tomentosa*; lanes 13-26 represent accessions of *Rosa canina* and lanes 27-46 represent wild rose accessions introduced from NBPGR, Shimla.

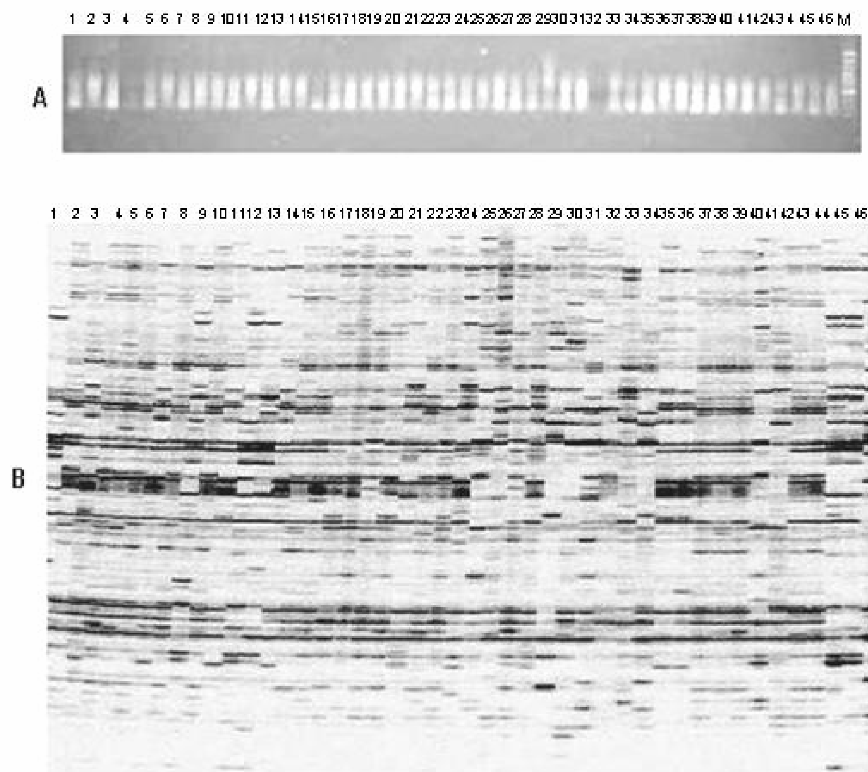

Fig. 3 (**A**) Restriction digestion (*Eco*RI/*Mse*I) products of DNA extracted by method 4 as visualized on 0.8% agarose gel stained with ethidium bromide. (**B**) Selective amplification with γ-P^{33}-ATP labeled *Eco*RI primer E-ACT and the 3 base *Mse*I primer M-CTC. Lanes 1, 9, 12, 13, 44, 45 represent *Rosa macrophylla*; lanes 4, 11, 14, 29, 30, 36 represent *Rosa tomentosa*; lanes 2-8, 10, 15-16, 37-39, 42-43 represent *Rosa canina* and 17-19, 20-34, 40-41, 46 represent accessions from NBPGR, Shimla.

nm ranged from 1.92 to 2.45, indicating only minor contamination of polyphenols and polysaccharides, whereas the $A_{260/280}$ nm ratio ranged from 1.68 to 1.94, indicating little contamination by proteins and macromolecules. *Eco*RI/*Mse*I completely digested the DNA (**Fig. 3A**) and good amplification profiles were obtained by RAPD, SSR and AFLP analysis (**Fig. 2B, 2C, 3A, 3B**). Thus the modified CTAB method of Xu *et al.* (2004) produced the greatest yield of quality DNA (900-1750 µg/g) compared to the other 3 methods. High concentrations of CTAB (3%), together with a high salt concentration (1.5 M) facilitated removal of polysaccharides and the high amounts of PVP (2%) and β-mercaptoethanol (4%) prevented oxidization of the secondary metabolites. These conditions have also been found to improve DNA quality in the extraction of cotton DNA (Zhang and Stewart 2000). The washing step before extraction removed contaminants, organic molecules, and excessive water. This step was also successfully applied in the isolation of RNA from fruit trees containing high levels of polysaccharides and polyphenols (Hu *et al.* 2002). Third, phenol was not used in the method. Polyphenols are easily oxidized in the presence of phenol and bind covalently to nucleic acid, which greatly reduces the DNA yield (Porebski *et al.* 1997; Thangjam *et al.* 2003). Also during the precipitation step, isopropanol in combination with sodium acetate was efficient in removing polysaccharides and secondary metabolites from DNA. In short, by using the procedure, we successfully isolated high-quality DNA from the 12 *Rosa* species.

High quality genomic DNA is critical for obtaining reproducible AFLP profiles. Compared with other molecular markers that just consist of a single PCR, AFLP process comprises five constitutive steps: DNA extraction, restriction/ligation, preamplification, amplification and electrophoresis. The restriction/ligation step makes the process more sensitive. The presence of secondary compounds can damage DNA; inhibit restriction ligation enzymes (Katterman and Shattuck 1983; Sharma *et al.* 2002) or *Taq* polymerase (Fang *et al.* 1992). Hence the DNA extraction protocol appears to be of most importance. Inhibition of restriction may result in incomplete digestion that produces variable AFLP banding patterns following amplification. The isolation protocols tried earlier on *Rosa* species (**Table 1**) that have yielded quality DNA amenable for restriction digestion, have utilized nuclei extraction buffers prior to lysis buffer, and have used various enzymes like proteinase K, Drislase, etc. for digestion of proteins and carbohydrates or have used silica gel membrane technology-based spin columns. Method 4 (modified CTAB method of Xu *et al.* 2004) also uses washing buffer (100 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 0.35 M glucose, 2% PVP (Mv 40000), 4% β-mercaptoethanol) prior to extraction buffer which is akin to nuclei isolation buffer and has resulted in removal of contaminants and organic molecules. Also DNA yields are higher as phenol is not used in this method. Although the quantity is not as critical as quality, 100-250 ng of genomic DNA per template is sufficient. But for marker screening and other molecular genetic analysis of crops higher yields are immensely beneficial. Also the method is simple and does not require enzymes for digestion of proteins or polysaccharides. Thus method 4 resulted in the isolation of intact, high quality DNA from 12 wild rose species that can be used for PCR- and endonuclease-based marker analysis.

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Appendix 1 *Rosa* accessions tested in this study.

***Rosa* species**	**№ of accessions tested**
Rosa alba	1
Rosa brunonii	4
Rosa bourboniana	1
Rosa bourboniana (transgenic)	8
Rosa canina	14
Rosa cathayensis	6
Rosa damascena	2
Rosa damascena x Rosa *bourboniana* F_1 hybrids	72
Rosa indica	1
Rosa macrophylla	6
Rosa moschata	5
Rosa multiflora	6
Rosa tomentosa	6
Rosa wichuriana	1
Wild accessions introduced from NBPGR, Shimla	20
Wild accessions from Palampur	4