

**Figure 4.** Paper chromatogram treated without (a) or with  $\text{NH}_3$  solution (b) and seen under UV transilluminator, showing qualitative and quantitative changes in flavonoid content in control and UV-B treated *G. indica* leaves.  $R_f$  values are also given.

extent anthocyanin, individually or in combination, may play an important role in the chloroplast movement. Further characterization and conformation of the nature of the phenolic compounds (with  $\lambda_{\text{max}}$  at 230 nm) accumulating under UV-B radiation by GC-MS and NMR will be helpful in elucidating the mode of action of UV-B radiation in chloroplast relocation.

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## Purification of DNA from chloroplast and mitochondria of sugarcane

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**We report here a method for the isolation of chloroplast DNA (ctDNA) and mitochondrial DNA (mtDNA) from leaf samples of 12 elite Indian sugarcane cultivars. This method includes isolation of organelles, purification by deoxyribonuclease treatment, organelle lysis, isolation of nucleic acid with phenol–chloroform and a final DNA purification using CTAB treatment. The protocol offers pure and completely restrictable ctDNA and mtDNA without nuclear DNA contamination. Inter-organellar DNA contamination was confirmed by PCR amplification using respective organellar DNA-specific universal primers. The ctDNA and mtDNA yield was approximately 7 and 5  $\mu\text{g/g}$  of leaf sample respectively. The method is useful for the isolation of ctDNA and mtDNA from plants that secrete high levels of polysaccharides and phenolic compounds.**

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To decipher the molecular complexities of organellar genome, gene structure and cytoplasmic variability, efficient preparation of pure, intact and nuclear DNA contamination-free chloroplast DNA (ctDNA) and mitochondrial DNA (mtDNA) are required. Initial attempts were made considering techniques/protocols available for the isolation of organellar DNA from plant source<sup>1-15</sup>. None of the protocols resulted in intact, sufficient yield or pure organellar genome preparation, though some of them are highly expensive, time-consuming and require long-term gradient centrifugation, while some are ineffective when applied to sugarcane leaf samples. This may be because sugarcane leaves are hard and fibrous, containing high levels of polysaccharides and phenolic compounds, which makes the isolation process difficult.

In the present communication, we report a novel method for the isolation of pure and restriction-digestible ctDNA and mtDNA from elite Indian sugarcane cultivars. We also describe separate protocols for the isolation ctDNA and mtDNA from sugarcane leaf samples using the available facility and by combining different steps from the available procedures based on biochemical principles of organellar purification and DNA extraction. The following protocol used for extraction of ctDNA and mtDNA from sugarcane leaves is novel, reproducible with maximum yield and recovers highly pure organellar genome.

In the present investigation we selected 12 diverse, widely cultivated, elite sugarcane cultivars with respect to their resistance/susceptibility to red rot disease, developed by the Sugarcane Breeding Institute (SBI), Coimbatore for molecular studies of organellar genome. These cultivars are maintained at the Bidar Sugar Factory farms (BSSK, Bidar). Healthy canes of 6-8-month-old plantations were selected for sampling. Plant materials were kept in a dark room at 4°C for 3 days to reduce the polysaccharide content<sup>16</sup>.

For chloroplast purification, leaf material from the second and third positions at the top was harvested and thoroughly rinsed with ice-cold distilled water and cooled to 0°C. Then 50 g of plant material was homogenized in a cold mortar with ice-cold extraction buffer (250 mM mannitol, 100 mM Tris buffer, 50 mM EDTA-Na<sub>2</sub>, 100 mM NaCl; add 0.2% bovine serum albumin, 0.1% cysteine and 1% PVP immediately before use) in a cold room. (All the subsequent steps of the protocol were carried out at 4°C, unless otherwise specified.)

The resulting homogenate was filtered through four layers of cheesecloth, followed by 100 µm nylon mesh and the filtrate was centrifuged at 1000 rpm (Sorvall RC5B) for 20 min to remove unlysed cells, cell debris and small fibres. The supernatant was recentrifuged for 20 min at 4500 rpm. The pellet was washed thrice with suspension

buffer (250 mM mannitol, 100 mM Tris buffer, 50 mM EDTA-Na<sub>2</sub>, 100 mM NaCl; add 0.2% bovine serum albumin, 0.1% cysteine and 1% PVP immediately before use) and resuspended in the same buffer using a soft paint brush. This suspension was layered onto the top of a 20-60% sucrose gradient (prepared in suspension buffer) and centrifuged at 48,000 rpm (Combi Plus, Dupont) for 4 h. The green interference containing chloroplast was collected using 24 gauge needle and incubated in a suspension buffer supplemented with 2 M NaCl for 15 min and then centrifuged at 5000 rpm for 15 min. The resulting pellet was washed repeatedly three times with suspension buffer and resuspended in MT buffer (400 mM mannitol; 50 mM Tris-HCl (pH-8.0)) using a soft paint brush.

For mitochondrial purification, the top unopened leaf roles were harvested and thoroughly rinsed with ice-cold distilled water and cooled to 0°C. Then 50 g of plant material was homogenized in a cold mortar with ice-cold MTEN buffer (400 mM mannitol, 50 mM Tris-HCl (pH 7.8), 25 mM EDTA-Na<sub>2</sub>, 1.5 M NaCl; add 0.2% bovine serum albumin, 0.1% cysteine and 1% PVP immediately before use) in a cold room. (All subsequent steps of the protocol were carried out at 4°C, unless otherwise specified.)

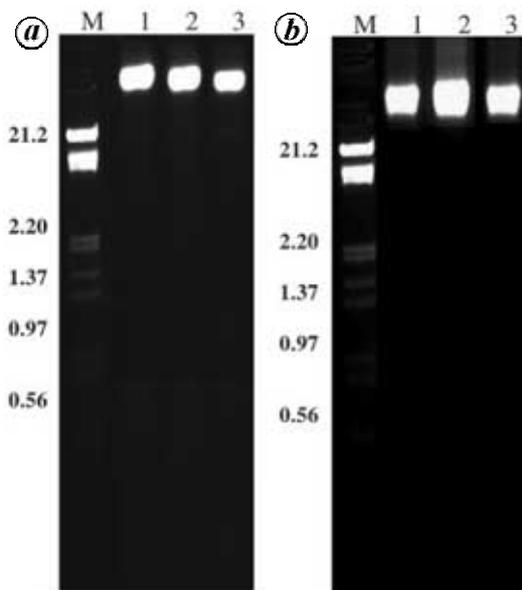
The resulting homogenate was filtered through four layers of cheesecloth, followed by 100 µm nylon mesh and the filtrate was centrifuged at 5000 rpm (Sorvall RC5B) for 20 min to remove plastids, unlysed cells, cell debris and small fibres. The supernatant was recentrifuged for 30 min at 14,000 rpm to sediment the mitochondria. Then the mitochondrial pellet was washed thrice with TENC buffer (100 mM Tris-HCl (pH 7.8), 50 mM EDTA-Na<sub>2</sub>, 100 mM NaCl; add 0.1% cysteine and 1% PVP immediately before use) and resuspended in MT buffer using a soft paint brush.

To the above chloroplast and mitochondrial suspensions, MgSO<sub>4</sub> was added to a final concentration of 20 mM and DNAaseI to a final concentration of 30 mg/g plant material. The mixture was incubated at 37°C for 1 h. After incubation, DNAase activity was arrested by washing with three volumes of NEDF buffer (1 M NaCl, 50 mM Tris-HCl (pH 8.0), 50 mM, EDTA-Na<sub>2</sub>, 2% DEPC and 50 mM NaF) and two more washings for complete removal of DNAase from the organellar suspension. Then the pellet was resuspended in TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA-Na<sub>2</sub>).

The organelles were lysed by incubating with 1% SDS and proteinase K to a final concentration of 10 µg/ml at 37°C for 1 h. After incubation, the mixture was treated with equal volumes of TE-saturated phenol and centrifuged at 10,000 rpm for 20 min at 4°C. Equal volume of chloroform/isoamyl alcohol (24:1) and 0.1 volume of 5 M sodium acetate was added to the upper layer and centrifuged at 10,000 rpm for 20 min at 4°C. The nucleic acids were precipitated by the addition of two volumes of chilled absolute ethanol and overnight incubation at -20°C. The

**Table 1.** Organellar-specific universal primers used for purity analysis

Primer	Gene	Primer sequence	Amplified product length (bp)
Chloroplast DNA-specific universal primers <sup>25</sup>			
a	<i>trnT</i> (UGU)	5'-CAT TAC AAA TGC GAT GCT CT-3'	789
b	<i>trnL</i> (UAA) 5' exon	5'-TCT ACC GAT TTC GCC ATA TC-3'	
c	<i>trnL</i> (UAA) 5' exon	5'-CGA AAT CGG TAG ACG CTA CG-3'	589
d	<i>trnL</i> (UAA) 3' exon	5'-GGG GAT AGA GGG ACT TGA AC-3'	
e	<i>trnL</i> (UAA) 3' exon	5'-GGT TCA AGT CCC TCT ATC CC-3'	460
f	<i>trnF</i> (GAA)	5'-ATT TGA ACT GGT GAC ACG AG-3'	
Mitochondrial DNA-specific universal primers <sup>26</sup>			
1	<i>Nad1B</i> (Forward)	5'-GCA TTA CGA TCT GCA GCT CA-3'	1180
2	(Reverse)	5'-GGA GCT CGA TTA GTT TCT GC-3'	
3	<i>Nad4</i> exon 1 (Forward)	5'-CAG TGG GTT GGT CTG GTA TG-3'	2100
4	(Reverse)	5'-TCA TAT GGG CTA CTG AGG AG-3'	
5	<i>Nad4</i> exon 2 (Forward)	5'-TGT TTC CCG AAG CGA CAC TT-3'	2840
6	(Reverse)	5'-AAC CAG TCC ATG ACT TAA CA-3'	



**Figure 1.** Agarose gel electrophoresis (0.8%) of chloroplast DNA (a) and mitochondrial DNA (b). M, DNA marker ( $\lambda$ DNA *EcoRI/HindIII* double digest); Lane 1, Co 62198; lane 2, CoC 85061 and lane 3, Co 86010.

DNA was recovered by centrifuging at 15,000 rpm for 30 min at 4°C. The pellet was washed with 70% ethanol, air dried and dissolved in TE buffer.

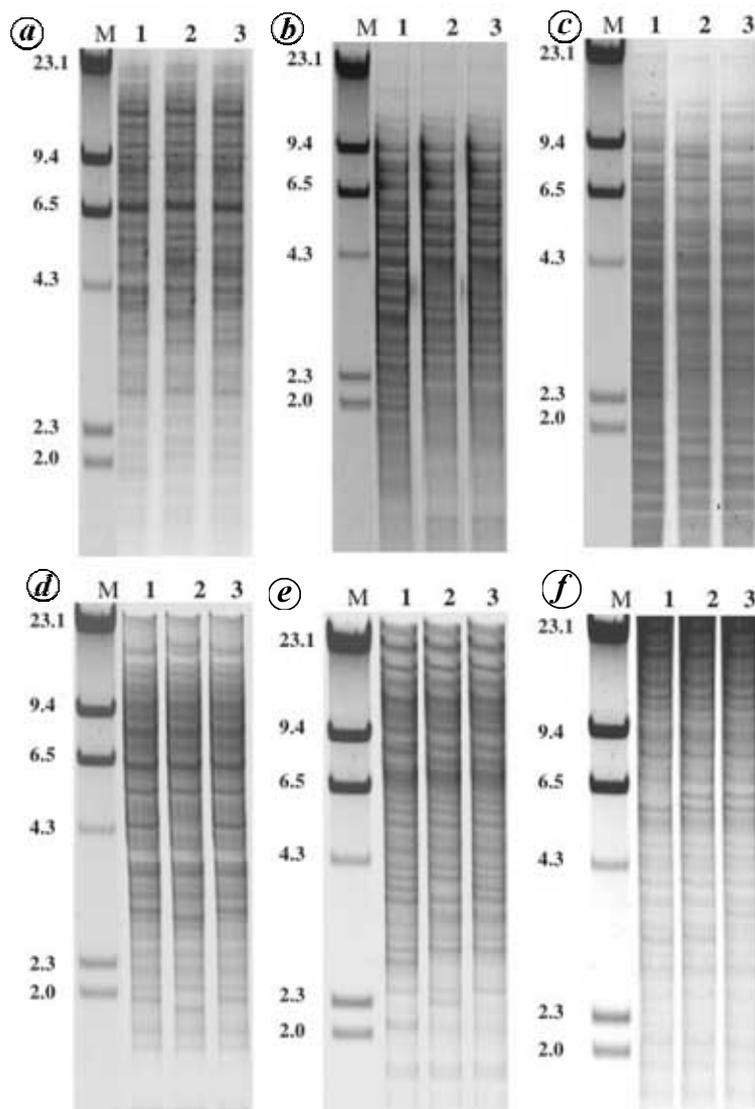
The DNA was made up to 600  $\mu$ l by adding the required amount of TE buffer. To this, 30  $\mu$ l of 10% SDS, 5  $\mu$ l of proteinase K (10 mg/ml) and 3  $\mu$ l of RNAase A (10 mg/ml) were added and incubated at 37°C for 1 h. After incubation, 100  $\mu$ l of 5 M NaCl and 80  $\mu$ l of CTAB/NaCl solution (10% CTAB, 0.7 M NaCl) were added and mixed thoroughly and incubated at 65°C for 15 min in a water bath. Equal volume of chloroform:

isoamyl alcohol (24:1) was added and vortexed for 2 min, and then centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was recovered from the above step and equal volume of phenol : chloroform : isoamylalcohol (25 : 24 : 1) was added and mixed thoroughly, and then centrifuged at 10,000 rpm for 15 min at 4°C. To the supernatant, 0.6 volume of isopropanol was added and incubated for 10 min at room temperature. DNA was recovered by centrifuging at 12,000 rpm for 20 min at 4°C and the pellet was washed with 1 ml of 70% alcohol. The pellet was air-dried by keeping the eppendorf in an inverted position for 10 min at room temperature and dissolved in 100  $\mu$ l of TE buffer.

Purity of the ctDNA and mtDNA was tested by treating all the DNA samples with six commercially available restriction enzymes, according to manufacturer's instructions, and further confirmed by amplifying with chloroplast and mitochondrial DNA specific universal primers (Table 1).

Most of the traditional methods of organellar DNA isolation techniques available in the literature involve isolation of intact organelles on continuous or discontinuous gradient centrifugation and removal of nuclear contamination or purification of organellar DNA by cesium chloride (CsCl) density gradient ultracentrifugation for long hours. This is expensive and time-consuming. Another approach for the isolation of organelles involves the differential centrifugation technique.

The pure and intact chloroplasts from sugarcane cultivars were recovered by sucrose discontinuous gradient ultracentrifugation at 48,000 rpm for 4 h at 4°C, with some modifications<sup>6</sup> in buffer composition and strength for complete removal of other organelles, cell debris and nucleus. A clearly separated chloroplast band between 35 and 45% sucrose gradient solution was obtained. It is difficult to separate the chloroplast, unlysed cells and cell debris using differential centrifugation due to little differ-

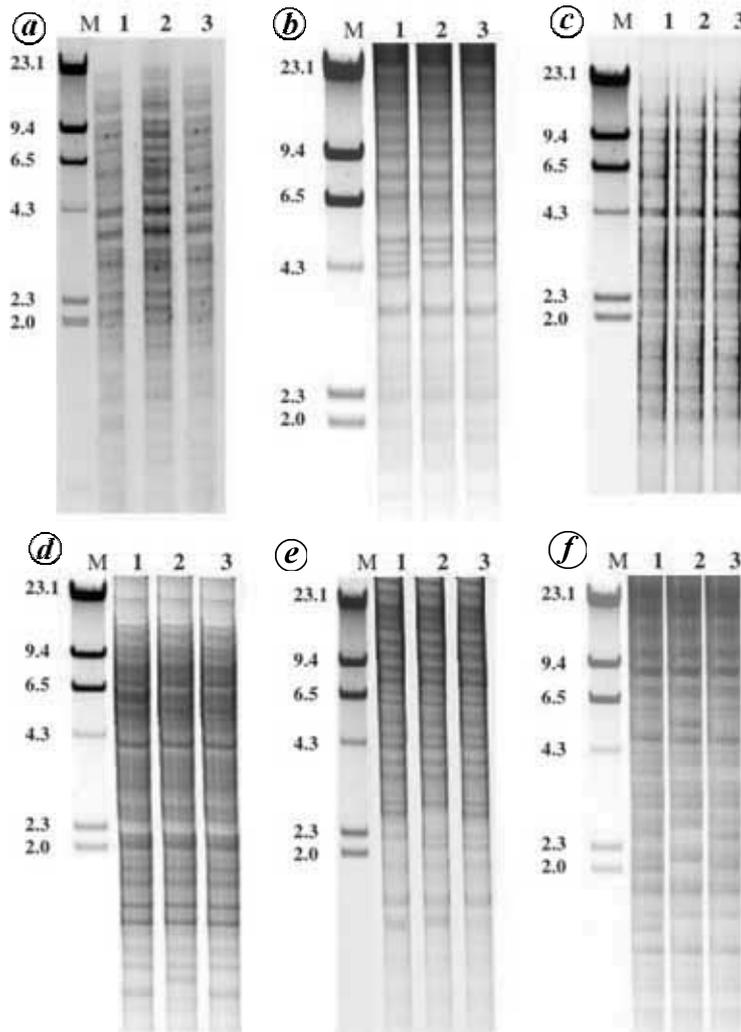


**Figure 2.** Restriction digestion pattern of chloroplast DNA of three sugarcane cultivars with six different enzymes on 0.7% agarose gel electrophoresis. *a*, *EcoRI*; *b*, *BamHI*; *c*, *HindIII*; *d*, *EcoRV*; *e*, *PstI*; *f*, *SalI*. M, Molecular marker ( $\lambda$ DNA *HindIII* digest). Lane 1, Co 62198; Lane 2, CoC 85061, and Lane 3, Co 86010.

ence in their sedimentation rate. A similar approach was reported for purification of chloroplasts from *Phalaenopsis* and *Doritis*<sup>6</sup>. Depending upon their buoyant density in discontinuous sucrose gradients, the organelles were separated by ultracentrifugation at 25,000 rpm for 50 min at 4°C and chloroplast band was observed between 30 and 60% interface<sup>5</sup>. The purified chloroplasts were washed repeatedly with suspension buffer in order to eliminate traces of nuclear DNA contamination and phenolic compounds.

The pure and intact mitochondrial preparations were obtained from sugarcane cultivars adopting modified<sup>3,17</sup> differential centrifugation methods with increasing ionic strength of the extraction medium. A similar technique was described for preparation of mtDNA from sunflower

seedlings or beet leaves and small amounts of potato tissue<sup>15,17</sup>. Use of high ionic strength alkaline buffers, BSA and cysteine protects the rapid inactivation of mitochondria from acidity, phenolic compounds and oxidation products<sup>14,15</sup>. Cysteine appears to be more efficient and less toxic than  $\beta$ -mercaptoethanol for protection against oxidation<sup>15</sup>. PVP adsorbs polyphenols, thereby preventing their interaction with DNA and organelles<sup>18</sup>. Homogenization was performed in cold room with pestle and mortar to achieve maximum recovery of intact chloroplast and mitochondria. In the present investigation, we have followed differential centrifugation for the elimination of chloroplasts and nuclear contamination from mitochondrial preparation in order to recover pure

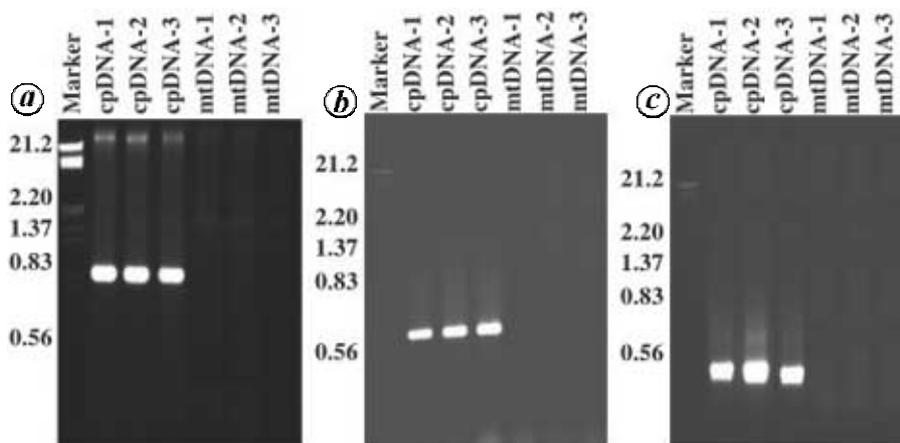


**Figure 3.** Restriction digestion pattern of mitochondrial DNA of three sugarcane cultivars with six different enzymes on 0.8% agarose gel electrophoresis. *a*, *EcoRI*; *b*, *BamHI*; *c*, *HindIII*; *d*, *EcoRV*; *e*, *PstI*; *f*, *SalI*. M, Molecular marker ( $\lambda$ DNA *HindIII* digest). Lane 1, Co 62198; Lane 2, CoC 85061, and Lane 3, Co 86010.

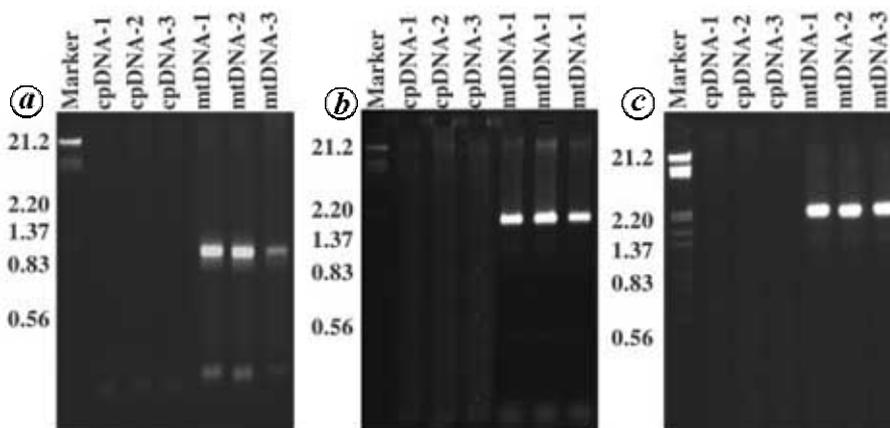
mitochondrial preparation. Similar reports are available on the use of differential centrifugation for the isolation of pure mitochondrial preparation. Mitochondria from tissue-cultured cell suspension culture of *Saccharum officinarum* and *Pennisetum americanum* were recovered<sup>19</sup> by differential centrifugation at 12,000 g. Differential centrifugation was used for the isolation of mtDNA from sunflower, with 12,000 rpm for 10 min at 4°C for pelleting the mitochondria<sup>14</sup>. Separation of mitochondria from chloroplast and removal of nuclear DNA contamination using differential centrifugation has also been reported<sup>20</sup>.

Near complete elimination of nuclear DNA contamination was achieved by incubating the chloroplast and mitochondrial suspension with DNAaseI at a final concentration of 30 mg/g plant material, and MgSO<sub>4</sub> at 20 mM for 1 h at 37°C. Both the DNAase and Mg/EDTA-Na<sub>2</sub> ratios

are essential for complete removal of nuclear DNA<sup>14</sup>. For magnesium ions we used MgSO<sub>4</sub>, because magnesium acetate can be replaced by magnesium chloride or magnesium sulphate<sup>2</sup>. After incubation, DNAase activity was inactivated by increasing the concentration of EDTA-Na<sub>2</sub> to 50 mM, because EDTA chelates the magnesium ions which are necessary for DNAase activity<sup>2</sup>. DNAase wash-off was performed in a special NEDF buffer, because EDTA alone cannot protect organellar DNA from cleavage during isolation, due to the presence of EDTA-activated DNAases. The NEDF buffer contains sodium fluoride (NaF) and DEPC, chemical inhibitors of DNAase and EDTA-activated DNAases respectively<sup>21,22</sup>. This takes care of traces of DNAase activity interference or degrade the DNA after lysis of the organelles. Similarly, NaF was used to inactivate DNAase activity for the isolation of ctDNA and mtDNA from sunflower<sup>14</sup>. In aqueous method



**Figure 4.** Purity of chloroplast DNA confirmed by PCR amplification of non-coding region with chloroplast DNA-specific universal primers. (a) 789 bp with primers a and b; (b) 589 bp with primers c and d; (c) 460 bp with primers e and f. M, DNA marker ( $\lambda$ DNA *EcoRI/HindIII* double digest). Lane 1, Co 62198; Lane 2, CoC 85061; Lane 3, Co 86010; Lane 4, Co 62198; Lane 5, CoC 85061, and Lane 6, Co 86010.



**Figure 5.** Purity of mitochondrial DNA confirmed by PCR amplification with mitochondrial DNA-specific universal primers. (a) 1.18 kbp with primers 1 and 2; (b) 2.1 kbp with primers 3 and 4; (c) 2.84 kbp with primers 5 and 6. M, DNA marker ( $\lambda$ DNA *EcoRI/HindIII* double digest). Lane 1, Co 62198; Lane 2, CoC 85061; Lane 3, Co 86010; Lane 4, Co 62198; Lane 5, CoC 85061, and Lane 6, Co 86010.

of ctDNA isolation from sunflower, DEPC was used instead of NaF to inactivate the novel EDTA-activated DNAases<sup>23</sup>. There are reports on the use of high concentration of EDTA alone to inactivate the DNAase activity<sup>15,20</sup>.

Purified chloroplast and mitochondrial suspensions were lysed with SDS and proteinase K. Then the DNA was isolated by standard phenol/chloroform method. The above isolated DNA was not sufficiently pure for restriction digestion analysis. Hence the DNA was further purified by CTAB/NaCl treatment to eliminate traces of polysaccharides, which will inhibit the enzyme activity during restriction digestion analysis<sup>24</sup>. DNA yield from sugarcane chloroplast and mitochondria was 6–8 and 4–6  $\mu$ g/g leaf material used respectively. The 0.8% (w/v) agarose

gel electrophoresis pattern of ctDNA and mtDNA is shown in Figure 1.

All the ctDNA and mtDNA were successfully digested with six different commercially available restriction enzymes (unpublished data). Here we show restriction digestion pattern of ctDNA and mtDNA of three cultivars with different enzymes (Figures 2 and 3). This indicates that isolated DNA from chloroplast and mitochondria of 12 elite Indian sugarcane leaves was pure and contamination-free.

When we amplified with ctDNA-specific universal primers (Table 1), there was no amplification in mtDNA samples (Figure 4), but amplification was observed in ctDNA preparation and vice versa for mtDNA (Figure 5). Further, the isolated ctDNA and mtDNA of 12 elite sug-

arcane cultivars was used for restriction digestion pattern analysis, RAPD, ISSR and phylogenetic analysis of chloroplast DNA based on their non-coding region nucleotide sequences (data not shown).

We have described a method for purification of ctDNA and mtDNA from elite Indian sugarcane cultivars. This protocol with minor modifications can be used for isolation of organellar genomes from other plant species that secrete high levels of phenolic compounds. This method offers several advantages. First, the protocol is suitable for plants containing high levels of phenolics and polysaccharides. Second, highly pure DNA is recovered in maximal amounts. Third, the protocol is scalable, and can produce required amounts of DNA according to the needs of the user. Finally, DNA can be stored for several years without any modifications in its properties.

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