

An efficient DNA isolation protocol for *Cymbopogon* species suitable for diverse downstream applications

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Abstract

Extraction of DNA from medicinal and aromatic plants is often problematic, since these plants contain high levels of secondary metabolites which interfere with PCR based downstream applications and restriction digestions. Removal of these secondary metabolites requires appropriate reagents for DNA isolation. This investigation optimised an efficient DNA isolation protocol for *Cymbopogon* species that yielded sufficient quantity of DNA and could be used for diverse molecular applications. The modified protocol was also compared with the two existing DNA extraction procedures for cost effectiveness, time efficiency and quality DNA recovery. The modified protocol yields good amount of DNA ranging from 76 to 90 µg/g of fresh tissues which was significantly higher in comparison to the other two protocols. A260/A280 ratio of the DNA obtained from the modified method ranged from 1.81 to 1.87 indicates purity of DNA and was also found to be suited for downstream applications such as restriction digestions. Subsequent RAPD, ISSR, SSR and barcode gene amplification analysis suggested that the DNA isolated by our modified method was suitable for various molecular research applications. The efficiency of this method in terms of lesser time requirement and cost effectiveness makes the present method a noticeable alternative for total cellular DNA extraction for *Cymbopogon* species and could be adoptable by the developing countries across the world.

Key words: CTAB, DNA fingerprinting, RAPD, ISSR, SSR, barcode, gene

Introduction

Isolation of good quality and quantity of DNA is a prerequisite for various molecular studies. Successful application of polymerase chain reaction (PCR) based downstream applications requires efficient recovery of good quality and quantity of DNA (Devi *et al.*, 2013). The cetyl trimethylammonium bromide (CTAB) method is the best method for extracting good quality of total cellular DNA from the plant tissues (Doyle and Doyle, 1990). Several CTAB based protocols were reported to extract pure and good quantity of DNA from different plant tissues (Khanuja *et al.*, 1999; Nischita and Ravishankar, 2013; Samantaray *et al.*, 2009; Sharma *et al.*, 2014). But, because of the complexity of secondary metabolites, none of the reported DNA extraction protocols is universally applicable for all plant species (Porebski *et al.*, 1997; Ribeiro *et al.*, 2007). Various commercial DNA isolation kits are also available in the market, however, the cost of these kits is a limiting factor for their use in developing countries (Ahmed *et al.*, 2009). Problems encountered in the isolation and purification of large quantity of qualitative DNA from medicinal and aromatic plant species includes degradation of DNA due to endonucleases activity, co-isolation of highly viscous polysaccharides and inhibitor compounds like polyphenols and also other secondary metabolites which may directly or indirectly interfere with the downstream enzymatic experiments (Weising *et al.*, 1995). Genus *Cymbopogon* is an important member of the family Poaceae (Gramineae), cultivated mainly for its essential oils. The essential oil of the species is used as preservative, pesticide, fungicide, bactericide, insect repellent and also as an ingredient in few medicines (Tyagi *et al.*, 1988). The presence of polysaccharides, polyphenols, essential oils and other secondary

metabolites hinder the recovery and purity of DNA (Porebski *et al.*, 1997) which demands complicated and time-consuming DNA isolation protocols in the species. Due to non availability of an efficient DNA isolation protocol for *Cymbopogon* species, the present study was focused on the development of a simple, rapid, reliable and inexpensive protocol for total DNA isolation. We also compared the newly developed method with the existing protocol (Khanuja *et al.*, 1999) as well as with one of the widely used commercial kit for its validation. The quality of the total cellular DNA obtained from the newly developed method was assured by allowing it to various downstream applications such as restriction digestion, RAPD (Random Amplified Polymorphic DNAs), ISSR (Inter-Simple Sequence Repeats), SSR (Simple Sequence Repeats) as well as for the plant barcode genes (*rbcl*, *matK*, *trnH-psbA* and ITS region) amplifications.

Material and methods

Plant material: Fresh and young leaves of six different species of the *Cymbopogon* such as *C. citratus* (DC.) Stapf, *C. flexuosus* (Nees ex Steud.) Wats., *C. martinii* (Roxb.) Wats., *C. nardus* (L.) Rendle, *C. pendulus* (Nees ex Steud.) Wats. and *C. winterianus* Jowitt ex Bor; two hybrids namely, CKP 25 [*C. khasianus* (Hack.) Staft ex Bor X *C. pendulus*] and Jamarosa [*C. nardus* X *C. jawarancusa* (Jones) Schult.] were collected from Field Gene Bank of ICAR-Directorate of Medicinal and Aromatic Plants Research, Anand, Gujarat (India) for optimising DNA isolation protocol. A minimum of ten replicates were taken from each species/ hybrids for this investigation.

Reagents and chemicals: Chemicals: Tris-Cl (1.0 M) pH 8.0, ethylene diamine tetra acetic acid (EDTA) (0.5 M) (pH 8.0), Cetyl

trimethylammonium bromide (CTAB), sodium chloride (NaCl), chloroform: isoamyl alcohol (24:1 v/v), polyvinylpyrrolidone (PVP, Mw 40,000) and β -mercaptoethanol (Amresco, USA).

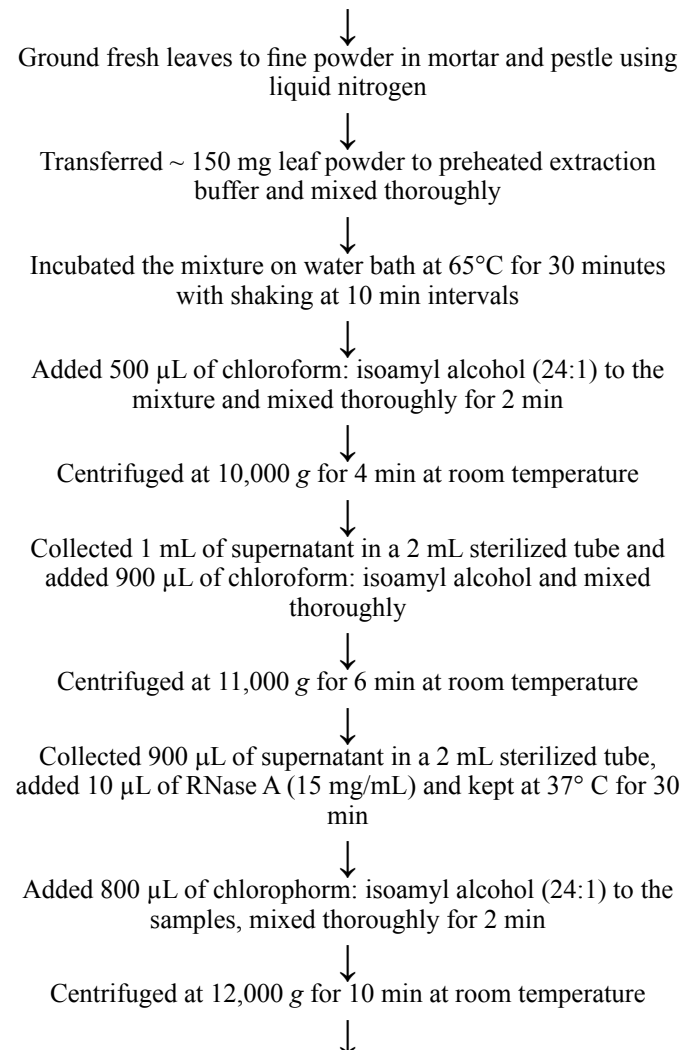
Restriction enzymes: *Eco* R I, *Hinc* II, *Hind* III, *Pst* I and *Alu* I (Pure gene, Genetix Biotech). Polymerase enzyme: *Taq* DNA polymerase (Bangalore Genei, India). RNase A (Bangalore Genei, India). Nucleotides: dNTPs (A, C, G, T) (MBI Ferment, Maryland, USA).

Primers: RAPD primer- (OPC 01 & OPP 15) (Operon Technologies Alameda, USA), ISSR primer- (AG)₈C & (CA)₈GT and SSR primer- CM 007 & CM 163 (Kumar *et al.*, 2007). (ISSR primers and SSR primers were synthesized from M/S Xcleris genomics, India), acetic acid (molecular biology grade) (Himedia), agarose (Bangalore Genei, India), ethanol, etc.

Extraction buffer: 100 mM Tris-Cl (pH 8.0), 20 mM EDTA (pH 8.0), 1.5 M NaCl, 4.0 % CTAB (w/v), 0.5% β -mercaptoethanol (v/v) and 2 % PVP (w/v) (both added immediately before use).

DNA extraction protocol: Standardization of quantity of chemicals (CTAB, PVP, β -mercaptoethanol, NaCl, RNase A) and time requirement of various processing (incubation, centrifugation, *etc.*) were done by different designed experiments. The best result was obtained as follows.

Took 1.2 mL of extraction buffer in a 2 mL sterilized centrifuge tube and preheated on water bath at 65°C for 2 min.



Collected 750 μ L of supernatant in a 2 mL sterilized tube, added 1 mL of chilled ethanol and mixed gently by inverting the tubes for 1- 2 min to precipitate DNA

Sedimented the DNA pellet by centrifugation at 5,000 g for 1 min

Washed the DNA pellet twice by 70% ethanol

Centrifuged the DNA at 12000 g for 2 min and carefully discarded the remaining ethanol

Air dried the DNA by keeping at room temperature for 3-5 min and dissolved the DNA in 150 μ L of TE buffer

Quantification and visualization of DNA: DNA quality and quantity were determined by measuring optical density (O.D.) at A260 and A280 with Nanodrop Spectrophotometer (M/S TECAN Infinite® 200 PRO NanoQuant, Switzerland). Five μ L of the isolated DNA was loaded on agarose gel containing ethidium bromide (0.5 μ g/mL) and were subjected to agarose gel (0.8 %) electrophoresis in 1X TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA) at 70 volt for 1 hour. Known standard (λ -DNA, 100ng/ μ L, Bangalore Genei, India) was also run to check the DNA quality and quantity. The gels were photographed using a Molecular Imager Gel Doc XR+ (Biorad, California, USA).

Restriction digestion: Total cellular DNA (1.5 μ g) of eight species/hybrids was digested with five restriction enzymes (RE) such as *Eco* RI, *Hinc* II, *Hind* III, *Pst* I and *Alu* I. The reactions were carried out in buffered conditions, incubated at 37°C for 3 hours following the manufacturer's instructions (Puregene, Genetix Biotech). The digested DNAs were run on 0.8% agarose gel electrophoresis along with standard (λ - DNA, 50 ng/ μ L) and marker (λ - Hind III digested and 50 bp DNA ladder; MBI Ferment, USA). The gels were photographed using a Molecular Imager Gel Doc XR+ (Biorad, California, USA).

RAPD, ISSR and SSR amplifications: PCR amplifications of the eight *Cymbopogon* DNA samples were carried out using two each of RAPD, ISSR and SSR markers to examine the quality of the isolated DNA in PCR applications. For RAPD and ISSR, the DNA amplification protocol (Williams *et al.*, 1990; Zietkiewicz *et al.*, 1994) was performed with necessary modifications according to the DNA sample and our laboratory settings. Each PCR amplification was carried out in 25 μ L reaction mixture containing 2 μ L of template DNA (25 ng/ μ L), 2 μ L of primer (5 pmol/ μ L), 0.3 μ L of dNTP mix (25 mM/ μ L) (MBI Ferment, Maryland, USA), 2.5 μ L of 10X assay buffer (10 mM Tris- Cl, pH- 8.0 with 15 mM MgCl₂, Bangalore Genei, India), 0.2 μ L of *Taq* DNA polymerase (5 U/ μ L) (Bangalore Genei, India) and final volume was made up with sterile double distilled water. For RAPD amplification, PCR cycles consisted of initial denaturation at 94 °C for 4 min, followed by 36 cycles at 92 °C for 1 min, annealing at 37 °C for 1 min and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. In order to perform ISSR amplification, initial denaturation was carried out at 94 °C for 4 min, followed by 36 cycles at 92 °C for 2 min annealing at 52 °C for 1 min. and extension at 72 °C for 2 min. followed by a final extension at 72 °C for 10 min. In case of DNA amplification in SSR

primers, reaction mixture and PCR thermal profiling were carried out as described by Kumar *et al.* (2007). The reproducibility of DNA profiles was tested by repeating PCR amplification with each of the selected primers for thrice. The amplification products were run on 1.6% agarose gel and the DNA profiles were documented by a molecular Imager Gel Doc XR+ (Biorad).

Bar code amplification and identification: Three DNA barcode genes of chloroplast (*rbcL*, *matK* and *trnH-psbA*) and one genomic DNA locus (ITS region) were amplified using the DNA isolated by the modified protocol. Nucleotide sequences of the four loci were retrieved from GeneBank of NCBI database. Forward and reverse primers were designed by software Primer3. Primers got synthesized by M/S Xcleris Genomics Lab, India. The PCR reaction mixture of 25 μ L contained 50 ng of template DNA, 1 \times PCR buffer, 1.5 mM of MgCl₂, 200 μ M of dNTPs, 0.25 μ M of each primer and 1 unit of Taq DNA polymerase. The PCR program used was as follows: an initial denaturation at 94 $^{\circ}$ C for 5 min, followed by 35 cycles at 94 $^{\circ}$ C for 1 min, standardized annealing temperature at 60 $^{\circ}$ C for 1 min, extension temperature at 72 $^{\circ}$ C for 2 min and final extension at 72 $^{\circ}$ C 10 min. A negative control run (without template DNA) was also performed at each time. PCR products were resolved in agarose gel (1.6%) electrophoresis. The gels were photographed by a molecular Imager Gel Doc XR+ (Biorad). The PCR products were purified using PCR cleanup kit (Nucleo-pore, Genetix Biotech), sequenced in M/S Xcleris Genomics Lab, India. The sequenced data were annotated and subjected to BLAST analysis (NCBI database) for confirmation of the barcode genes.

Results and discussion

The newly developed protocol resulted in yield of impurity free, good quantity DNA ranging from 76 to 90 μ g/g of fresh tissue

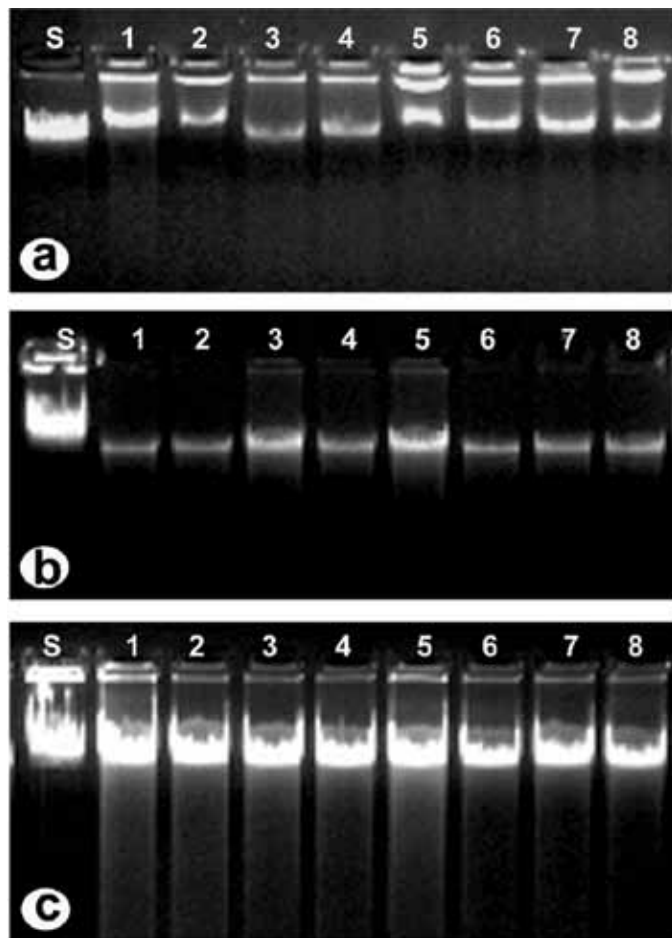


Fig. 1. Electrophoresed gel (0.8% agarose) image of total DNA extracted from of *Cymbopogon* spp. using (a)- existing protocol, (b)- Qiagen mini plant kit and (c)- New modified CTAB protocol. Lane 1 to 8- *Cymbopogon* spp., S- standard (λ - DNA, 100 ng/ μ L).

Table 1. Quantitative estimates of DNA concentration as revealed by Nanodrop Spectrophotometer in *Cymbopogon* spp.

<i>Cymbopogon</i> spp.	Replications (Yield of DNA in μ g/g of fresh leaves)										Mean \pm SD	CV (%)
	1	2	3	4	5	6	7	8	9	10		
<i>C. citratus</i>	89.15	88.49	89.94	92.12	91.25	90.05	89.15	88.79	91.56	92.85	90.34 \pm 1.52	1.68
<i>C. flexuosus</i>	85.45	86.57	90.74	91.21	87.02	89.24	86.76	92.42	88.18	87.85	88.54 \pm 2.29	2.58
<i>C. martinii</i>	90.12	87.54	88.23	87.21	90.0	89.54	85.47	89.68	88.36	91.12	88.73 \pm 1.69	1.90
<i>C. nardus</i>	82.23	78.54	78.05	79.89	77.42	76.5	85.94	79.1	78.13	80.87	79.67 \pm 2.77	3.48
<i>C. pendulus</i>	76.53	79.56	79.15	77.25	79.10	82.12	81.18	77.15	76.87	80.0	78.89 \pm 1.91	2.42
<i>C. winterianus</i>	76.12	81.25	73.56	79.47	80.74	76.85	79.5	74.47	77.23	78.12	77.73 \pm 2.57	3.30
CKP25	89.37	82.24	85.4	91.23	85.54	84.8	85.36	85.57	90.0	90.45	87.00 \pm 3.01	3.46
Jamarosa	75.25	76.78	79.65	75.47	76.7	75.23	75.36	76.0	78.15	71.55	76.01 \pm 2.13	2.80

Table 2. Comparison of DNA isolation protocols based on DNA yield, recovery, OD 260/280 ratios, processing time and materials cost

<i>Cymbopogon</i> Species	DNA yield (μ g/gm of fresh tissue)			Recovery (%/ mg of fresh tissue)			OD ratio (260nm/280nm)			Processing time (min)			Cost per 100 reactions *(INR)		
	E	K	M	E	K	M	E	K	M	E	K	M	E	K	M
<i>C. citratus</i>	39	50	90	3.9	5.0	9.0	1.77	1.90	1.81						
<i>C. flexuosus</i>	56	42	89	5.6	4.2	8.9	1.75	1.96	1.85						
<i>C. martinii</i>	35	60	89	3.5	6.0	8.9	1.49	1.88	1.87						
<i>C. nardus</i>	39	48	80	3.9	4.8	8.0	1.67	1.89	1.86	250	40	100	9640	30640	3246
<i>C. pendulus</i>	44	46	79	4.4	4.4	7.9	1.46	1.89	1.85						
<i>C. winterianus</i>	54	64	78	5.4	6.4	7.8	1.61	1.88	1.87						
CKP25	42	54	87	4.2	5.4	8.7	1.69	1.88	1.83						
Jamarosa	41	48	76	4.1	4.8	7.6	1.66	1.92	1.86						

Abbreviations: E: Existing protocol (Khanuja *et al.*, 1999), K: DNeasy Plant Mini kit (Qiagen), M: Modified protocol. *INR= Indian Rupee

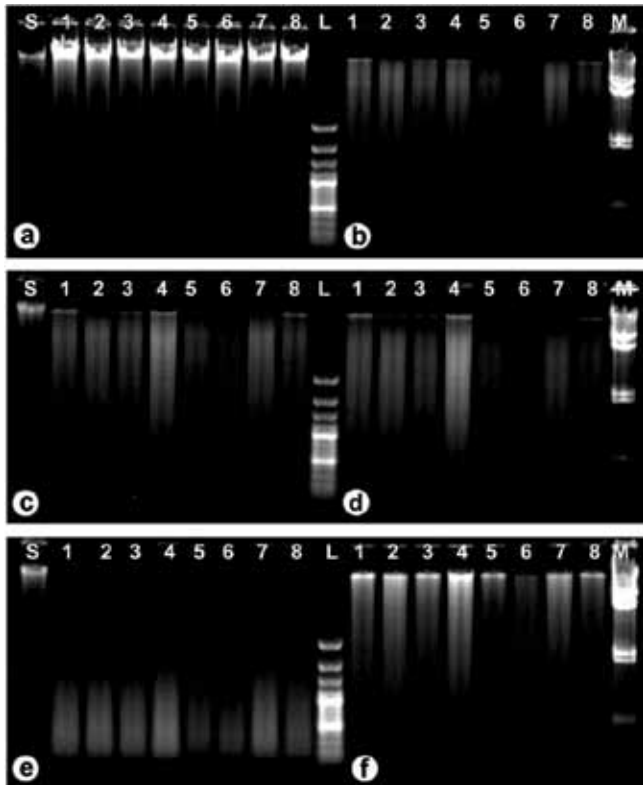


Fig. 2. Electrophoresed gel (0.8% agarose) image of restriction digested DNA of *Cymbopogon* spp., (a)- undigested control DNA, (b)- digested with *Eco* RI, (c)- digested with *Hinc* II, (d)- digested with *Hind* III, (e)- digested with *Pst* I and (f)- digested with *Alu* I. Lane 1 to 8- *Cymbopogon* spp., S- standard (λ - DNA, 50 ng/ μ L), L- 50 bp DNA ladder, M- λ - DNA

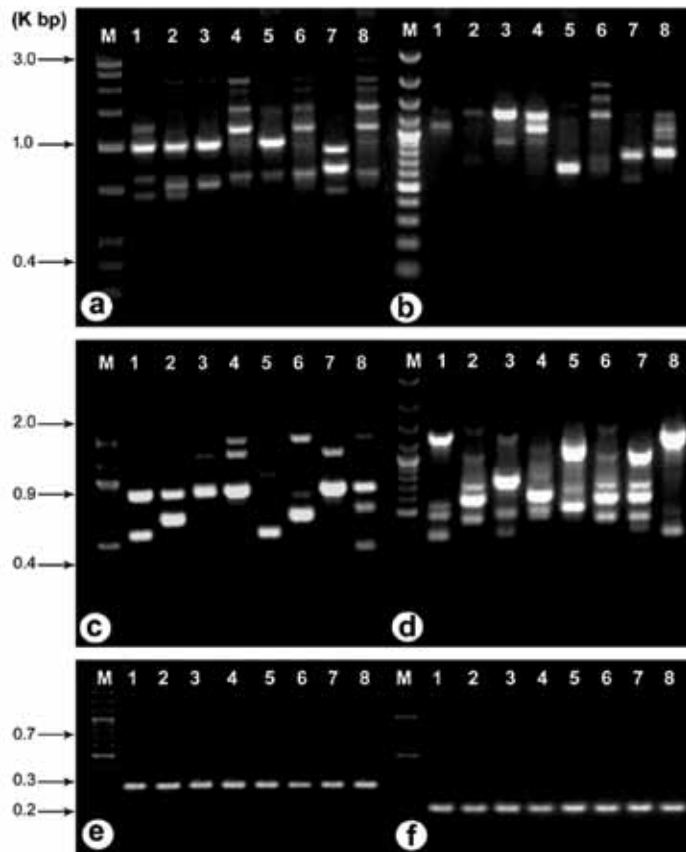


Fig. 3. PCR profiling of *Cymbopogon* spp. (a) & (b)- RAPD profiling with primer OPC 01 & OPP 15, (c) & (d)- ISSR profiling with primer $(AG)_8C$ & $(CA)_8GT$ and (e) & (f)- SSR profiling with primer CM 007 & CM 163, respectively. Lane 1 to 8- *Cymbopogon* spp., M- 100 bp DNA ladder.

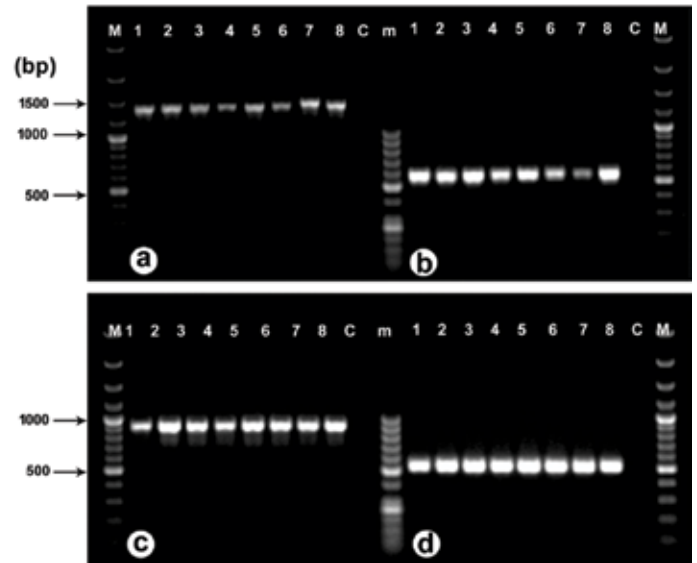


Fig. 4. Amplification of bar code region in of *Cymbopogon* spp. (a)- *rbcL* gene (~ 1350 bp), (b)- ITS 1 & ITS 2 region (~ 600bp), (c)- *matK* gene (~ 940 bp) and (d) *trnH-psbA* gene (~ 550 bp). Lane 1 to 8- *Cymbopogon* spp., c- negative control, M- 100 bp DNA ladder, m- 50 bp DNA ladder.

(CV% = 1.68- 3.48) (Table 1). The OD ratio (A260/A280) was in the range of 1.81 to 1.87 which indicated the purity of the DNA. Yield and purity of DNA from each samples were found to be superior in our protocol as compared to the DNA isolated by the existing protocol and the kit based method (Table 2, Fig. 1 a, b & c). The processing time was also approximately less than half in the modified protocol as compared to the existing protocol. The existing protocol is maxi based isolation which consumes ~3 times more chemicals and adds to the overall cost of isolation as compared to our modified protocol which is a mini isolation protocol (Table 2). The new protocol resulted in extraction of DNA ranging from 6.5 μ g to 9.0 μ g per gram of fresh tissue which is sufficient for 300 to 400 PCR reactions. In contrast, Qiagen Plant DNA extraction kit required less time (~ 40 minutes), however DNA yield was lower and it was comparatively more expensive (approximately 9.44 times) (Fig. 1 b; Table 2). In addition, the modified protocol required only chloroform, which eliminates the necessity of phenol, making the method more user friendly (Agbagwa *et al.*, 2012).

We also optimised DNA precipitation step (addition of ethanol in single step) instead of double step, which increased the yield of intact DNA and also reduced the ethanol consumption and time requirement. Further, the use of high concentration of CTAB (4%) aided in yielding high concentration of DNA and the use of PVP and β -mercaptoethanol were helpful in removing the polyphenols, essential oils and other impurities. The quantity of DNA recovered in new protocol (percentage per mg of plant material) was sufficiently high as compared to the kit and also with the existing protocols and the increase was up to 56 to 120% more (Table 1&2). The restriction enzymes digestion analysis confirmed the good quality of the isolated DNA samples through our modified protocol (Fig. 2 a to f). This indicated that the isolated DNA was amenable to further processing in cloning as well as in DNA fingerprinting experiments. Additionally, the efficiency of isolated DNA in PCR amplifications was also demonstrated by RAPD profiling with two random primers (Fig. 3 a & b), ISSR profiling with two different 3' anchored primers (Fig 3 c & d) and

SSR profiling with two reported primer combinations (Fig. 3 e & f). The results of the amplification in barcode genes followed by BLAST analysis indicated that the isolated DNA contained not only genomic DNA but also the chloroplast DNA (Fig. 4 a to d) of the species. The efficiency of this method in terms of fewer steps, less time consumption and lower cost made the present method a highly acceptable alternative for the extraction of high quality and higher amount of total cellular DNA from *Cymbopogon* spp.

In conclusion, the method described here is a rapid, simple and inexpensive protocol for the extraction of total cellular DNA isolation from *Cymbopogon* spp. that could be used in a wide range of downstream applications. In addition, this method may also be suitably adoptable for extraction of total cellular DNA from the other medicinal and aromatic plants. Hence, the protocol would be more useful for the developing countries where cost is a prohibiting factor for molecular research.

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