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Full Length Research Paper

A simple method of DNA extraction from coffee seeds suitable for PCR analysis

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High quality genomic DNA was successfully extracted from coffee seeds using a simple protocol devoid of liquid nitrogen or freeze-drying and proteinase K. The isolated DNA was quantified using spectrophotometer and using agarose gel electrophoresis. The DNA was free from polysaccharides, polyphenols, RNA and other contaminants. The quantity of DNA ranged from 180 to 630 µg/g of seed powder. Quality of DNA was confirmed by digestion using *EcoRI, HindIII* and *PstI* restriction endonucleases and complete digestion was observed. PCR with random decamer primers and consensus primers of mitochondria and chloroplast DNA and PCR-RFLP revealed the suitability of the DNA for PCR based marker techniques including diagnostics.

Key words: Coffee seed, DNA extraction, RAPD, PCR-RFLP, molecular diagnostics.

INTRODUCTION

Coffee is one of the most important agricultural comm.-odities in international trade. Coffee represents an important source of income for millions of people in coffee growing countries in Asia, Africa and Latin America. Many countries depend on the export of this product to buy goods and equipments. Commercial coffee cultivation relies upon two coffee varieties; *Coffea arabica* (*Arabica coffee*) and *Coffea canephora* (*Robusta coffee*). Arabica produces high quality coffee and contribute about 70% of total world coffee production. Robusta produces inferior coffee compared to Arabica. Highly priced varieties would benefit from diagnostic tests for authenticity and provenance in this commodity, as coffee is liable to be adulterated with the addition of inferior quality of coffee, husks or chicory (Martellosi et al., 2005). Since coffee is traded as beans, it is essential to develop a diagnostic tool using the seed material for identifying the adulteration that will benefit the consumers worldwide. Such a diagnostic tool will be useful for the developing countries that are involved in coffee growing. In addition, several consuming countries particularly in Europe and USA are now insisting on GM-free coffee while importing the produce from growing countries. These demand a diagnostic protocol for differentiating between GM and non-GM coffee beans.

Further, leaf samples, which are generally used for DNA isolation for molecular analysis, need to be freeze-dried using lyophilization or liquid nitrogen before transporting them to far off places (Agwanda et al., 1997). Since the availability of liquid nitrogen is limited in many of the developing countries where coffee is grown in very interior provinces, seeds make an ideal material as they can be easily transported from one place to another place. Several molecular techniques now currently available for identifying species or varieties within a blend and provide valuable tool for guaranteeing consistent quality. This makes coffee seed as one of the important material for molecular marker studies.

Like many other tree plant species, coffee contains high amounts of polysaccharides, polyphenols and various secondary metabolites such as alkaloids, flavonoids and phenols which usually interfere during DNA isolation. In addition to this, co-isolation of highly viscous polysaccharides along with DNA was the major problem encountered during coffee DNA isolation. Several methods have been reported for minimizing the DNA extraction steps and cost (Berthomieu and Meyer, 1991; Edwards et al., 1991). But none of these protocols as such were found suitable for DNA isolation from coffee

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Table1. DNA content in seed samples of various coffee genotypes.

S/N	Genotype	Parentage	Isolated DNA (µg/g seed)
1	Sln.5A	Devamachi x S.881	582.0
2	Sln.6	S.274 x Kents	396.0
3	Sln.9	HdeT x Tafarikela	350.0
4	S.795	S.288 x Kents	366.0
5	Hybrid 1	S.795 x Sln.9	300.0
6	Hybrid 2	Sln.9 x S.795	417.5
7	Sarchimor	Villarsachi x Hybrido de Timor	360.0
8	Cauvery	Caturra x Hybrido de Timor	330.0
9	Columbian Catimor	Catimor from Columbia	246.0
10	C X R	*C. congensis* x *C. canephora*	295.0
11	S.274	Indian Robusta selection	487.5
12	Perpurascens robusta	Natural Arbugogov mutants	184.0
13	Tree coffee hybrid	*C. arabica* x *C. liberica* (putative hybrid)	636.0
14	Agaro	Exotic-Ethiopian collection	337.0
15	Cioccie	Exotic-Ethiopian collection	283.0
16	Tafarikela	Exotic-Ethiopian collection	252.0

seed. A rapid DNA isolation protocol was reported by Kang et al. (1998) but the same protocol was not suitable when large amount of DNA was required. Moreover, proteinase K used in their protocol was not found suitable and it also increases the cost of the protocol. Kobayashi et al. (1998) developed a simple and efficient DNA isolation protocol suitable for woody plants. However, they used liquid nitrogen for isolation of DNA which is again a limiting factor for many laboratories. We, therefore, developed a simple, efficient and rapid protocol for isolation of DNA from coffee seeds suitable for molecular studies.

MATERIALS AND METHODS

Coffee fruits of different varieties (Table 1) were collected from the experimental plot of Central Coffee Research Institute during the month of November – December 2004. The fruits were pulped using a hand operated baby pulper and parchment samples were sun dried up to 10% moisture level. Parchment samples were hulled in a baby huller and clean green coffee samples were taken for experimental analysis. Fifty gram of dried seeds of different coffee varieties were crushed in a mill and powdered by using a domestic grinder. The powder was sieved using thin mesh and only finely ground powder was kept in refrigerator until DNA extraction.

DNA isolation protocol

One gram of finely sieved seed powder was taken and homogenised in 10 ml of freshly prepared extraction buffer (200 mM Tris-HCl pH 8.0, 50 mM EDTA, 2.2 mM NaCl, 2% hexadecyltrimethylammonium bromide (CTAB), 0.06% sodium sulphite, 50 mg sarcosine, 50 mg poly vinylpyrrolidone, MW 40,000 (PVP) (w/v), 100 µl mercaptoethanol (added just before use) and mixture was transferred to 30 ml centrifuge tubes. The tubes were incubated at 65°C for 1 h with intermittent shaking and swirling at 15 min interval, and cooled to room temperature. An equal volume of chloroform-isoamylalcohol (24:1) was added and mixed gently by inversion for 10 min and centrifuged at 6000 rpm for 15 min at room temperature. The supernatant was carefully transferred to a new 30 ml tube and DNA was precipitated by addition of two-thirds volume of ice-cold isopropanol and 1 ml of 5 M sodium chloride by incubating overnight at –20°C.

The sample was centrifuged at 5000 rpm for 5 min at 4°C followed by 8000 rpm for 15 min. The DNA pellet washed with 80% ethanol and air dried and resuspended in 500 µl of TE buffer (10 mM Tris HCl, 1mM EDTA pH8.0) and transferred to 1.5 ml microfuge tube. RNA contamination was removed by incubating DNA with 5 µl RNaseA (10 mg/ml stock) at 37°C for 60 min.

The dissolved DNA was extracted with equal volume phenol : chloroform : isoamylalcohol (25:24:1, v/v/v) at 8000 rpm for 15 min at 4°C. The aqueous layer was transferred to fresh 1.5 ml microfuge tube and reextracted with an equal volume of chloroform: isoamylalcohol (24:1) at 8000 rpm for 15 min at 4°C.

The supernatant was transferred to a fresh 1.5 ml microfuge tube and DNA was reprecipitated with equal volume of chilled ethanol and half volume of 7.5 M ammonium acetate and kept at –20°C for one hour. DNA was spooled using a thin Pasteur tip, dried and dissolved in 200 µl TE buffer.

Quantity and quality of DNA

The yield of DNA per gram of seed material extracted was measured by using UV spectrophotometer at 260 nm and A280 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260/280 nm. DNA concentration and purity was further tested by running the samples on 0.8% agarose gel and compared with the known standard Lambda DNA marker.

The suitability of isolated DNA for molecular analysis was further determined by digesting the genomic DNA with *EcoRI, Pst I* and *HindIII* restriction endonucleases. The reaction mixture was carried out in 20 µl volumes containing 4 µl of DNA, 2 µl of 10X assay buffer, 2 µl of BSA (10 mg/ml) and 8 units of restriction enzymes at 37°C overnight. The digested DNA was electrophoresed on 1.0% agarose gel and visualized using Syngene Gene snap UK gel doc system.

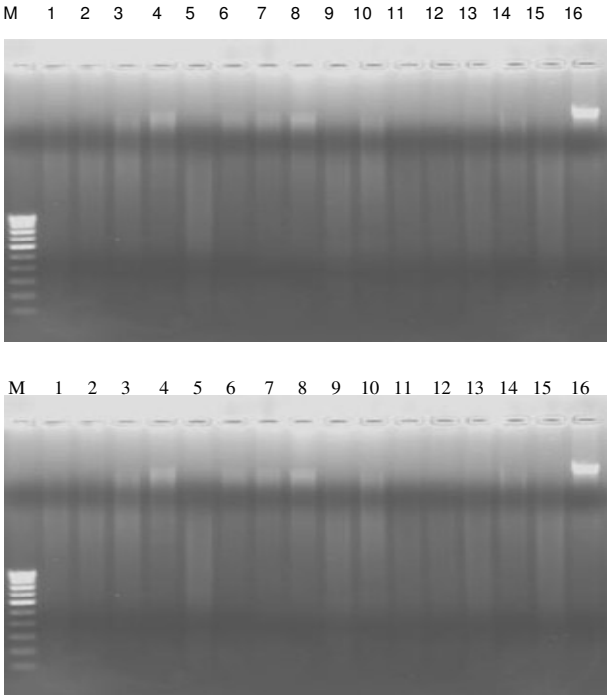

Figure 1. Restriction digestion of the seed genomic DNA with *EcoRI,* (1-5) *Hind III* (6-10) and *Pst I* (11-15). M = Marker (100 bp ladder); 1, 6 and 11 = Sarchimor; 2, 7 and 12 = Cauvery; 3, 8 and 13 = S.274; 4, 9 and 14 = Agaro; 5, 10 and 15 = Cioccie; 16 = undigested control.

PCR amplification using random primers (RAPD)

Polymerase chain reactions for amplifications using random Operon primers (5'-GGGTAACGCC-3' and 5'-GGGACGTTGG-3' were carried out in a 25 µl volume for all DNA preparations. The PCR reaction mixture contain 2.5 µl 10X buffer with 15 mM $MgCl_2$ (Fermentas), with 0.25 mM each of dNTP, 0.3 µM of the primer, 0.5 unit of *Taq* DNA polymerase (Bangalore Genei,) and 50 ng of template DNA. PCR reaction was performed in Palm Cycler (Corbett Research) using the following profile with initial denaturation of 4 min at 95°C followed by 40 cycles of 1 min at 95°C, 1 min at 38°C and 2 min at 72°C with final extension at 72°C for 10 min and a hold temperature of 4°C at the end. Amplified PCR products were electrophoresed on an agarose gel (1.5%) prestained with ethidium bromide (0.5 µg/ml) in 1X TAE buffer and visualized using Syngene Gene snap UK as described earlier.

PCR amplification using mitochondria and chloroplast specific primers

PCR amplification using chloroplast and mitochondria specific primers were carried out in a 25 µl volume reaction mixture containning 2.5 µl 10X buffer with 15 mM $MgCl_2$ (Fermentas), with 0.25 mM each of dNTP, 0.3 µl of each primer (reverse and forward), 1 unit of *Taq* DNA polymerase (Bangalore Genei), 50 ng of template DNA. The reaction profile employed for amplification were initial denaturation of 4 min at 95°C followed by 40 cycles of 45 s at 94°C, 45 s at 50-58°C for (depending on the primer) and 2 min at 72°C for extension with a final extension step at 72°C for 10 min. The Amplified PCR products were electrophoresed on an agarose gel (1.5% v/v) pre stained with ethidium bromide (0.5 µg/ml) in 1X TAE buffer and visualized as described earlier.

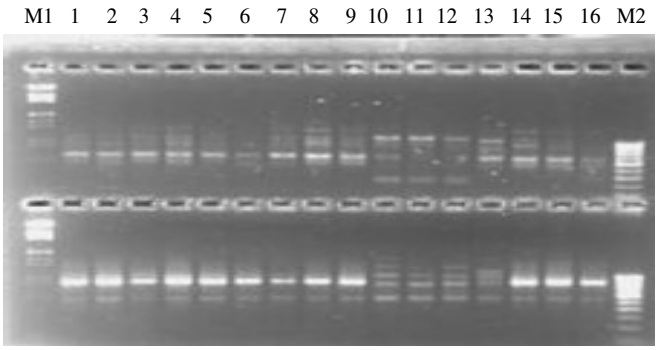

Figure 2. RAPD profiles with primer GGGTAACGCC (upper lanes) and GGGACGTTGG (lower lanes). Lane M1, Lamda double digests; 1. Sln5A; 2. Sln 6; 3. Sln9; 4. S.795; 5. S.795 x sln.9; 6. Sln9 x S.795; 7. Sarchimor; 8. Cauvery; 9. Columbian Catimor; 10. C x R; 11. S.274; 12. Perpurascence Robusta; 13. Natural hybrid; 14. Agaro; 15. Cioccie; 16. Tafarikela; and M2, 100 bp ladder.

PCR RFLP

PCR products (5-10 µl) were digested in a reaction mixture of 20 µl volume containing: 2.0 µl of 10X assay buffer, 0.2 µl BSA 10 mg/ml) and 4 unit of each restriction enzyme (Fermentas) separately and digestion was carried out for 3 h as per the suppliers' instruction for each enzyme. The digested DNA was electrophoresed on 1.5% agarose gel and visualized as described earlier.

RESULTS AND DISCUSSION

We were able to obtain high quality DNA free from polyphenols and polysaccharides from the coffee seeds. When spooled out of solution, the DNA is clear or white; there is no visible coloration. The A_{260}/A_{280} ratio of the DNA ranges from 1.7 to 1.85 indicating the isolated DNA is free from protein and RNA contamination. Yields of the DNA varied with the cultivars and ranged from 180 to 630 µg for 1 g of seed powder (Table 1). The extracted DNA showed no visible RNA contamination, as determined by agarose gel electrophoresis and could be digested completely by *EcoRI, Hind III* and *PstI*, which indicated the suitability of the DNA for RFLP and AFLP analysis (Figure 1). The extracted DNA stored at –20°C for more than one year showed no deleterious effects and could be amplified. In order to check the efficiency and reliability of the method, we first amplified the seed DNA of coffee using random primers. The PCR amplification was robust (Figure 2) and on par with leaf DNA. (Data not shown)

We further tested the suitability coffee seed DNA for amplification of mitochondrial and chloroplast DNA using consensus primers (Taberlet, 1991; Dumesure et al., 1995; Dumolin et al., 1997). In both the cases (Figures 3 and 4), as expected a single robust band was obtained and this confirmed the earlier observations of various authors in different crops (Pharmawati et al., 2004; Vettori et al., 2004; Achere et al, 2004). Further the PCR

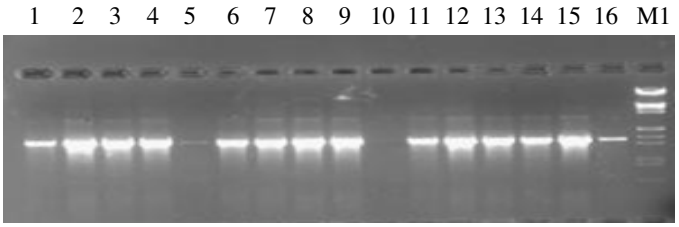

Figure 3. Seed DNA amplified with mitochondrial specific primer (mt-*nad1B – nad1C* region). Lane M1, Lamda double digests; 1. Sln5A; 2. Sln 6; 3. Sln9; 4. S.795; 5. S.795 x sln.9; 6. Sln9 x S.795; 7. Sarchimor; 8. Cauvery; 9. Columbian Catimor; 10. C x R; 11. S.274; 12. Perpurascence Robusta; 13. Natural hybrid; 14. Agaro; 15. Cioccie; and 16. Tafarikela.

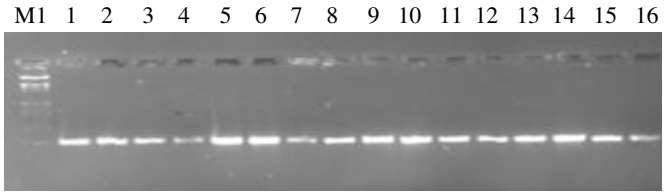

Figure 4. Seed DNA amplified with chloroplast specific primer (trnL intron region). Lane M1, Lamda double digests; 1. Sln5A; 2. Sln 6; 3. Sln9; 4. S.795; 5. S.795 x sln.9; 6. Sln9 x S.795; 7. Sarchimor; 8. Cauvery; 9. Columbian Catimor; 10. C x R; 11. S.274; 12. Perpurascence Robusta; 13. Natural hybrid; 14. Agaro; 15. Cioccie; and 16. Tafarikela.

product of the trnL intron region of *cp*DNA could be successfully cut with *TaqI* restriction enzyme for assessing the variability in chloroplast DNA (Figure 5).

A diagnostic test was carried out by amplifying the DNA of *C. arabica* and *C. canephora* varieties separately as well as by mixing the samples of both the varieties. In all the *C. canephora* samples, a 1 kb fragment was obtained which was absent in the *C. arabica* samples. In the mixed samples, *C. canephora* specific band was amplified. A clear differentiation pattern between *C. arabica*, *C. canephora* and their mixture was obtained by PCR amplification of DNA using random primers (Figure 6).

This clearly suggests the importance of molecular marker studies in diagnostics and validated the present protocol.

In conclusion we have developed an easy and reliable DNA isolation protocol from coffee seeds. The amount and quality of the DNA is suitable for PCR marker studies as well as sufficient for RFLP analysis. The protocol could be scaled up or down depending on the quantity of the coffee seeds available as well as the requirement of DNA material for particular molecular analysis. This method will be useful for molecular diagnostic studies in this important crop species and could also be extended to extract DNA from the seeds of other plant species as well.

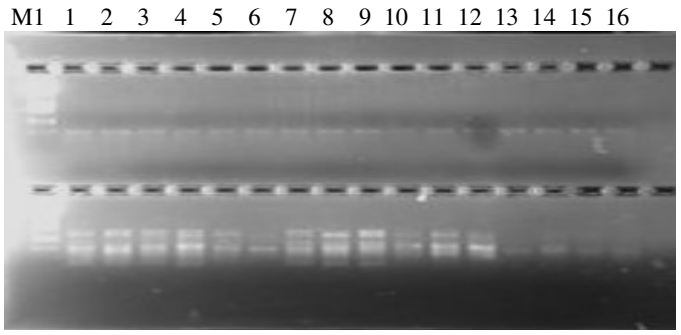

Figure 5. RAPD profiles (upper lanes) and restriction digestion of the *cp*DNA *trnL* int region using *Taq I* enzyme (lower lanes). Lane M1 Lamda, DNA double digest; 1. Sln5A; 2. Sln 6; 3. Sln9; 4. S.795; 5. S.795 x sln.9; 6. Sln9 x S.795; 7. Sarchimore; 8. Cauvery; 9. Columbian Catimor; 10. C x R; 11. S.274; 12. Perpurascens Robusta; 13. Natural hybrid; 14. Agaro; 15. Cioccie; and 16. Tafarikela.

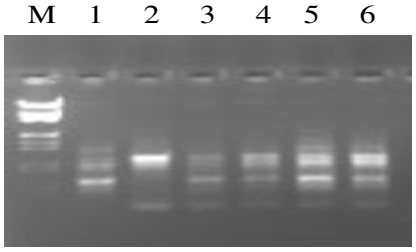

Figure 6. Diagnostic tool for the mixture using primer (GGGTAACGCC). Lane M, Lambda DNA double digest (*EcoRI* and *HindIII*); 1. Sln5A; 2. S.274; 3. Sln 9 and S.274; 4. S.795 and S.274; 5. Sln. 5A and S.274; 6. S.274 and Columbian Catimor.

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