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Genetic diversity of NMT gene in coffee using SCAR marker

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Abstract
Coffee tree belongs to the genus *Coffea* comprising of two main cultivated species *C arabica* L. and *C. caenophora* and third sparsely grown species *C liberica* in some parts of Africa. Importantly, many of these diverse species hybridize readily with each other and thus are a rich and valuable source of desirable genetic variability for improvement of cultivated coffee germplasm. But breeding coffee has been difficult because of a variety of genetic and environmental factors. Also, the caffeine in coffee is a major source of health problems in the developed and in the developing nations. One solution would be to develop coffee varieties which have less caffeine or caffeine free, retaining their flavour using the gene manipulation techniques or by using molecular markers. It has been found that three genes namely, 7-methyl xanthosine synthase (CAXMT1/CmXRS1), two theobromine synthases (CAMXMT1/CTS1 AND CAMXMT2/CTS2) and caffeine synthase (CADXMT1/CCS1) are responsible for the synthesis of caffeine in the coffee plant. Hence an attempt has been made to construct gene specific markers (SCAR marker) and confirm the presence of genetic diversity for NMT gene in coffee in few of the South Indian varieties. Also the genetic diversity of NMT gene both in coding and non coding region was chequed. The genetic diversity recognized by the SCAR marker is very similar, although the diversity is very narrow for the NMT gene.

Keywords: genetic diversity, NMT gene, SCAR marker

Introduction
Caffeine is a plant alkaloid known to have sensory and stimulatory effects, which is present in beverages such as coffee and tea. Caffeine protects young leaves and fruits from predators and that caffeine released by the seed coat prevents the germination of other seeds.

The biosynthetic pathway of caffeine in coffee plants is predicted to involve successive synthesis from purine precursors such as AMP and GMP through multiple steps catalyzed by several enzymes. In the final stage of caffeine synthesis, methylation of xanthosine to yield 7-methylxanthosine. After removal of its Rib residue, the 7-methylxanthine is methylated at 3-N position to produce theobromine. This is further methylated at 1-N position to give 1, 3, 7 trimethylxanthine (caffeine).

Consumers concerned about possible adverse effects of caffeine consumption will welcome this development towards caffeine free drinks that retain their flavor. The increasing demand for decaffeinated coffee and tea has resulted from the occasional side effects of caffeine, which include palpitations, gastrointestinal disturbances, anxiety, tremor, increased blood pressure and insomnia. At present, decaffeination process depends on supercritical fluid extraction with carbon dioxide to avoid introducing toxic residues from extraction solvents. However, this operation is expensive, flavors and aromas are lost. The modern day better approach would be to manipulate the gene responsible for the caffeine synthesis or choosing / isolating a variety having reduced amount of caffeine synthesis.

So present investigation was to isolate the NMT gene from different varieties of South India and compare the genetic variability for NMT gene using molecular markers. This could further help in gene manipulation of NMT gene by utilizing the sequences.

Materials and Methods
Plant Material: Inbred lines of *C. arabica* were collected from coffee growing areas in Karnataka as given in table 1. Young leaves were collected from plants of each cultivar, frozen in liquid N_2 and kept at -80ºC until use. *C. canephora* cultivar was used as outgroup accession.

Genomic DNA extraction: Total genomic DNA was extracted from frozen young leaves according to Doyle and Doyle protocol (1987) [3] and Paillard *et al*. (1996) [7] protocol.

Table 1

Sl. No	Accessions	Species	Place collected from
1	S5	Arabica	Ooty
2	S10	Arabica Catuai	Chikmangalur
3	S11	Arabica	Lalbagh
4	S12	Arabica	Lalbagh
5	C*R	Robusta	Lalbagh

PCR Amplification: SCAR (Sequence Characterized Amplified Regions): A total of 40ng of each DNA sample was used in PCR reactions for SCAR amplification. The designed primers were used for amplification. PCR reactions were set as follows in a final volume of 25 µl; 0.1 mM/l, 2mM/l Mgcl 2, 0.5UmM/l primer, IX reaction buffer (10mmoles/L TRIS-HCL and 50nmol/l KCL), 0.25U Taq polymerase. Samples were subjected to 45 cycles under the following conditions:

1min at 94^{O}C, 45s at 35^{o}C and 1.5 min at 72^{o}C. Amplified fragments were separated according to size on 1.5% agarose gel and stained with ethidium bromide. Gel documentation and fragment size determination by the software Image Master Total Lab.

PCR Amplification: Various combinations of primers were tried to get the largest possible amplicons to NMT gene. The PCR reaction was carried out by combining the following reaction components in 25µl reaction volume.10X reaction buffer for XT-TAQ system (Chromus Biotech) contains 20mM $MgCl_2$, Tris, KCl and Gelatine. The contents of the tube were mixed by a brief spin in a micro centrifuge.

Gel Electrophoresis: Agarose, TAE 50X buffer (24.2 g Tris base,5.71 ml glacial acetic acid, 10ml of 0.5M EDTA),1kb DNA marker, Ethidium Bromide (10 mg/ml), Gel casting boat, Mini gel apparatus, power supply.

Designing of primers: Primers were designed using Primer3, AutoDimer software.

Methods Used

Isolation of DNA was done using Doyle and Doyle (1987) [3] **and Paillard *et al*. (1996)** [7] **method**

PCR Amplification

The PCR amplification was carried out in the Department of Biotechnology, RVCE using PCR Thermo cycler TaKaRa. PCR was carried out on 25 µl volume (23µl master mix+ 2 µl of template DNA) in a thermocycler for amplification. Amplification reaction mixture of 25 µl contains 10X reaction buffer (20mM Tris- Hcl, 20mM KCl and 20ml MgCl2, 10mM dNTPs, 1 unit of Taq polymerase, 1200µM of primer and 120ng of template genomic DNA.

Analysis of PCR product by agarose gel electrophoresis

8µl aliquot of the PCR product was analysed by agarose gel (1.2%) electrophoresis as described below:

1. The boat was sealed with an adhesive tape and the comb was placed for the wells.
2. 0.72g of agarose was added to 60ml of the 0.5X TAE buffer. The mixture was heated on a hot plate to dissolve the agar. The solution was cooled to 50^{o}C and poured into sealed boat.
3. 2µl of ethidium bromide was added to the gel just before the solution was poured on the boat. The gel was allowed to polymerize.
4. The comb and adhesive tape were removed and the gel was placed in the electrophoresis tank with sufficient volume of 0.5X TAE buffer to cover the surface of the gel.
5. PCR reaction sample and the standard DNA size marker were loaded in the wells. Electrophoresis was carried out at 50 volts till the dye reached 3/4th of the gel.
6. The gel removed from the tank was examined on a UV transilluminator and documented using Gel Documentation system (JH BIO).

Primer Design: The design of the primers was done using Primer 3 software and Auto dimer software.8 sets of primers were selected for PCR amplification process. The following is the list of primers.

Table 2

SCAR F1	GTGATTGAATTGGGGATTGG	SCAR R1	GTCTAGGGGATTCGGTTCGT
SCAR F2	GACAAAGTTGGCCAGGAAGA	SCAR R2	CGATCCTATTTTGCGTCCAT
SCAR F3	GTTGCTGCCAAGCTTCTACC	SCAR R3	CAATCCCCAATTCAATCACC
SCAR F4	TGGCCAGGAAAAGAAGAATG	SCAR R4	CGGGGAAGAGTCTGCTGTAG
AJ250259-F	CCCTCCCTGCCAGAAGAAGC	AJ250259-R	CTGCGAGGTGCTTATTGAAG
SCAR-F5	TCCGAATTCCGTAGAAAGAGCCAG	SCAR-F5	AGACAGACATTGTCGGATGC
CS	AGCTCCAAGAAGTCCTGCAT	CS	ACTCGGATAGAACCCCAACC
XMTF (promoter sequence)	AGCAGTCGCAATTCGATTGTC	XMTR	ACGACAATACCCGAAGACC

Results

- **UV quantification of DNA:** Ratio OD 260/OD 280 was determined to assess the purity of the sample. Doyle & Doyle (1987) [3] method showed that the ratio OD 260/OD 280 was highest in *Coffea arabica* S10 i.e., 1.40, followed by 1.29 in *Coffea arabica* S12. *Coffea arabica* S5 gave an OD 260/ 280 ratio of 1.21. Lowest ratio OD 260/280 was obtained in *Coffea arabica* S9 i.e., 1.13 and in *Coffea canephora* C*R i.e., 1.036. Paillard method gave highest OD 260/ 280 ratio in *Coffea canepora(robusta)* C*R followed by 1.74 in *Coffea arabica* S10. *Coffea arabica* S12 gave an OD 260/280 ratio of 1.69. Lowest OD 260/ 280 ratio was obtained in *Coffea arabica* S5 i.e. 1.63 and in *Coffea arabica* S9 i.e. 1.55(Table 1).
- **DNA Concentration:** DNA Concentration was calculated using the relationship formula for double stranded DNA. DNA concentration for *Coffea arabica* S5, *Coffea arabica* S9, *Coffea arabica* S10, *Coffea Arabica* S12, *Coffea canepora* (*robusta*) C*R was found to be 1.3757µg/µl, 1.757µg/µl, 2.3757µg/µl, 1.007µg/µl, 1.7µg/µl DNA respectively(Table 2).

PCR Amplification

PCR amplification was carried out for 8 sets of primers with 5 DNA samples (Fig 1). Microsatellite primers gave good results with 1% agarose and the bands obtained were 100bp in length. The second gels that we acquired results were from primer pairs of SCAR F1 and SCAR R1 that showed polymorphism in the second well (Fig 2). The gel percentage in which the bands were seen was 1% agarose. The third gel whose products were acquired from primer pairs SCAR F2 and SCAR R2 also showed very slight polymorphism in the second well (Fig 3).

Bands were obtained below 100bp, optimization of the gel

electrophoresis and PCR seemed necessary to be undertaken for better resolution. Light bands were postulated to be an effect of less $MgCl_2$. Hence, the $MgCl_2$ content was increased by additional 7mM concentration. The annealing temperature was decreased to 57ºC.This was done to allow better binding of the primer on to template DNA. Agarose percentage was gradually increased to 1.8% as the size of the DNA was approximately 100bp. The last gel from the primers XMTF1 and XMTR1 were designed to isolate promoter sequence of the NMT gene by Sathyanarayana *et al*. (2005) [8] and these primers showed bands in the wells (Fig 4). Optimization results also showed better band resolution and also a slight variation in the gene sequence.

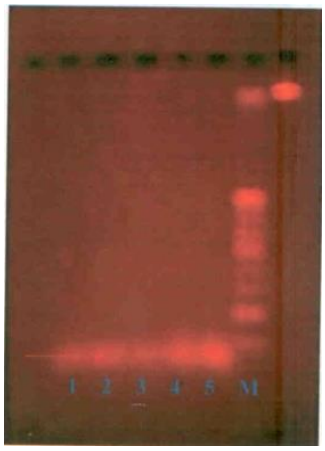

Fig 1

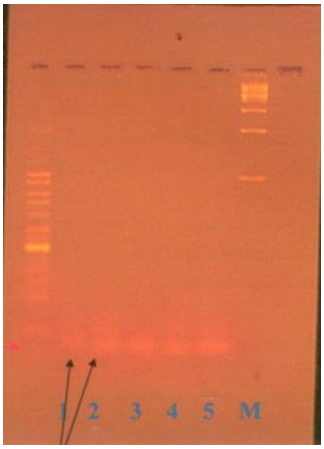

Fig 2

Fig 3

Fig 4

Discussion

Extraction of plant DNA from coffee

Young leaves which grew second, just below the apical leaf tip, were taken for extraction of plant DNA as they were considered to have minimal polyphenolic production (Sathyanarayanan *et al*., 2005) [8].

Xin Xu *et al*. 2005 [10] used the Robust CTAB activated method which uses neither liquid nitrogen nor freeze drying for initial grinding of the tissue. This method reduces the times of chloroform extraction by directly using potassium acetate to remove the contaminating proteins in the same step. These alterations significantly minimize time and the use of laboratory materials. The amount of DNA that they have achieved is about 5-20ng of high molecular weight DNA per centimeter of fresh leaf tissue. A ratio of absorbance at 260nm to that at 280nm of 1.9-2.3 indicated insignificant levels of contaminating proteins and polysaccharides. The extraction of DNA obtained from liquid nitrogen was considered to be the better technique as can be observed from our analysis as well. Tumoru Sera *et al*. (2003) [9] showed that their quantity of DNA from *Coffea arabica* varieties were upto 10 ng/ml, with slight modifications in the Doyle and Doyle (1987) [3] protocol. This was the use of MATAB instead of the conventional CTAB.

Mitja Krizman *et al*. (2006) [5] used a robust CTAB activated charcoal protocol for plant DNA extraction that gave a good amount of Coffee DNA of 7ng/ml. This method although, highly efficient, was rejected for the sole reason of charcoal forming dark coloration in the samples. These were feared to obscure the purity of the DNA.

Our UV Spectrophotometer readings coincide with an average of 1.6µg/µl of DNA that we quantified in our samples. These are proof that the DNA that we acquired, coincides with many of the corresponding authors whose DNA concentrations coincide with similar ranges. One of the smallest problems in extraction of plant DNA from coffee is that the coffee plant is very high in its phenolic content. The polysaccharides in the plant are also in a considerable amount as can be seen by the UV Spectrophotometer readings by the Doyle and Doyle (1987) [3] protocol. The gel electrophoresis of the extracted DNA for confirmation analysis showed sharp bands which confirmed purity of our samples.

The method of extraction by Paillard *et al.* helps in the removal of majority of the polyphenolics and the polysaccharides. The detergents CTAB and SDS used in the protocol in the steps of lysis buffer and extraction buffer along with multiple washing with Chloroform: Isoamyl alcohol (24:1) aided in the process of removal of the polysaccharides. Also addition of PVPP during the grinding of the young leaves helped absorb the polyphenolics as soon as the leaf tissue was lysed.

The designed primers were cross checked with the Auto Dimer Software which showed any absence of hairpin looping or even primer pair complexation. For the sake of analysis we had used pre deigned primers from Sathyanarayanan *et.al* (2005). The primer pairs of A250255 and A250259, pairs of XMTF1 and XMTR1 were pre synthesized. The other primers were designed from the coding sequence region of *Coffea caenophora* accession number AY918125.1.

PCR amplification and product analysis

The first sequences that gave us results were the microsatellite regions. PCR protocol followed by Satyanarayana *et al.* (2005) [8] for the isolation of the promoter for NMT genes was the protocol followed by us. Mirian Perez Maluf *et al*.2006 [6] showed that polymorphism occurred between *C. canephora* and *C. arabica* cultivars. But not many polymorphisms were observed between different varieties in the same species. The overall polymorphism degree detected by all systems is very low compared to similar analysis in other plant species (Lee,

1995). Most polymorphisms occur between *C. arabica* and *C. canephora*. These results agree with other studies on genetic diversity of *Coffea* commercial cultivars (Lashrmes et al., 1993; Anthony *et al.,* 2001) [1], which in general, report low genetic diversity of *Coffea*.

This was probably the result of a narrow genetic basis of the cultivated coffee, as very few accessions from African countries, such as Yemen and Ethiopia, the geographical origin of *C. arabica*, were introduced and are the basis of all breeding programs. Also, *C. arabica* is an autogamous species, what contributes to low heterozygosity levels.

The polymorphisms that were observed in the second wells by the SCAR markers, SCAR F1, R1 and the pairs SCAR F2, R2 also correspond to the same feature. The products obtained were close to 100bp mark. This showed that we either needed to have increased the concentration of agarose or have optimized the PCR protocol to get better bands.

The optimization of the protocol included an increase in the $MgCl_2$ by an additional 7mM concentration. Also, the annealing temperature was decreased to 57ºC. The agarose gel percentage was increased to a gradual 1.8% and then observed. The gel 4 thus resulted in sharp bands that also showed very slight variations among the first variety and the last four varieties.

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