

Development of 'Assam' type tea specific SCAR marker from RAPD products

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Received 5 February 2013; revised 22 May 2013; accepted 10 August 2013

Tea is one of the most important plantation crop in India, accounting for two-third of global tea production. However, the genetic diversity of tea is narrowing down because of the large-scale use of vegetatively propagated clones and massive uprooting of age-old bushes. The present study was carried out to develop DNA based marker for characterization of tea germplasms. A RAPD marker (811 base pair) putatively specific to 'Assam' type tea was identified and converted to sequenced characterized amplified region (SCAR) marker by cloning and sequencing the specific RAPD product. A pair of primers from SCAR marker so produced amplified a 640 bp product specifically identifying the 'Assam' type tea clones from others. The marker was validated and found to be useful for identifying the 'Assam' type tea clones easily and economically.

Keywords: Assam type tea, *Camellia assamica*, cultivar-specific, RAPD, SCAR

Introduction

Tea is one of the oldest and most popular non-alcoholic soft beverages known across the world. Botanically tea is *Camellia sinensis* (L.) O. Kuntze under the family *Theaceae*¹. It is one of the most important plantation crops grown in India, accounting for two-third of global tea production, which earns a substantial foreign exchange. The commercial grown tea cultivars are recognized under three taxon, viz., *C. sinensis*, *C. assamica* and *C. assamica* ssp. *lasiocalyx*, and distinctly categorized as 'China', 'Assam' and 'Cambod' type, respectively². South-East Asia is believed to be the original home of tea³. Tea has received much attention for its attractive aroma, pleasant taste and numerous medicinal benefits, and has been socially and habitually consumed by people since 3000 BC⁴. Many secondary metabolites, such as, polyphenols, alkaloids (caffeine), vitamins, polysaccharides, volatile oils and minerals, are contained in tea leaves⁵. Tea polyphenols specifically include catechins, flavanones and phenolic acid, and these compounds may account for up to 30 per cent of the dry weight⁶. Thus tea has an immense contribution to the health and employment opportunity in India.

The important factor affecting the Indian tea among others is the ageing of tea bushes, leading to declining quality and productivity. The bushes are

also becoming more susceptible to pests and diseases, necessitating uses of high dosage of pesticides leaving residual effect on the made tea. Moreover, in different region in India, Assam in particular, seed materials are collected from different sources to develop the original tea plantation. Present day cultivation completely depends on some newly developed/selected vegetatively propagated clones, thus narrowing down the genetic diversity. Fast uprooting of the age-old plantations in the tea estates lead to further loss of important genetic materials, which are fundamental for tea breeding and biotechnology. Study of inheritance in tea is complicated by a number of factors, such as, its perennial nature, open pollination, inbreeding depression, etc. Therefore, germplasm characterization of tea at molecular level can be a benchmark for varietal improvement, preserving the intellectual property right, development of molecular passport and taxonomic classification⁷. Molecular markers like RFLP, RAPD, SCAR, AFLP and SSR can aid in shortening the breeding cycle and marker assisted selection process.

PCR based marker system, viz., RAPD (random amplified polymorphic DNA)^{8,9}, in which short oligonucleotides of arbitrary sequences are used, is relatively easy, inexpensive and fast technique. However, reproduction of RAPD markers often suffers from lack of specificity and sensitivity to the reaction conditions¹⁰. More recently, robust sequence characterized amplified region (SCAR) markers that were designed based upon the nucleotide sequence of

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specific RAPD-PCR markers were found stable, easy to use and inexpensive for single locus assay in different crops¹¹⁻¹⁴. SCAR marker is developed with a pair of longer primers compared with universal primers. The longer length (approx 25 bp) and higher temperature (65°C) of SCAR primers make the reaction more specific, reproducible and stable¹⁵⁻¹⁷. Elucidation of type specific molecular fingerprints shall be fundamental for tea breeding. Further, it would help in identification of cultivar used by the manufacturer in a particular brand of tea. In the present study, we used three distinct types of *Camellia* species (China, Assam & Cambod) and developed SCAR marker for taxonomically distinct 'Assam-type' tea from a species-specific RAPD amplicon.

Materials and Methods

Plant Materials

A total of 10 different accessions representing 3 distinct types of tea were included in the present study, viz., TV1 (Assam China hybrid), TV5 (Assam type), TV6 (Assam type), TV7 (China hybrid), TV16 (Assam hybrid), TV17 (Assam hybrid), TV18 (Cambod type), TV19 (Cambod type), TV29 (Cambod triploid) and TV30 (Cambod type). Fresh tender young shoots were collected from the Experimental Tea Field of Assam Agricultural University, Jorhat. The fresh leaves were stored in deep freezer (–80°C) till further processing.

Isolation of *Camellia* Genomic DNA

Total DNA was extracted from the tender leaf as described by Dellaporta *et al*¹⁸ with little modification. The extraction buffer contained 100 mM Tris-HCl, 50 mM EDTA, 500 mM NaCl, 1% SDS, 10 mM β-mercaptoethanol and 0.5% polyvinyl pyrrolidone (PVP), pH 8.0. Following a single chloroform-isoamylalcohol (24:1) extraction, the precipitated DNA was resuspended in sterile TE (10 mM Tris, 1 mM EDTA, pH 8.0), followed by RNase (10 µg/mL) treatment to remove RNA. DNA concentration was determined by taking absorbance at 260 nm in a spectrophotometer.

Identification of Specific RAPD Amplicon

Twenty random primers (OPA-01 to OPA-20) (Operon Biotechnologies GmbH, Cologne, Germany) were screened initially to identify well reproducible bands in all the accessions. Amplification of DNA from the 10 *Camellia* accessions was carried out using a single primer at a time.

Polymerase chain reaction (PCR) was carried out in a 25 µL reaction mixture containing 50 ng of DNA, 1 U of *Taq* DNA polymerase enzyme, 1 µL of dNTP (10 mM), 1× *Taq* DNA polymerase buffer with 1.5 mM MgCl₂ and 20 pmol decanucleotide primer. Amplifications were carried out in a DNA thermal cycler (Perkin Elmer/Gene Amp PCR system 2400) using following PCR parameters: 94°C for 1 min; 45 cycles at 92°C for 1 min, 40°C for 1 min and 72°C for 1 min; and a final extension at 72°C for 7 min. PCR products were subjected to agarose gel (1.5% w/v) electrophoresis in 0.5× TBE buffer, along with 500 bp DNA ladder (Bangalore Genie, India) as size markers. DNA was stained with ethidium bromide and photographed under UV light. The amplicon, which was monomorphic to *C. assamica* but absent in other 8 *Camellia* germplasms was identified.

Cloning and Sequencing of RAPD Products

The putative marker amplified by random primer OPA-10 of approx 811 bp was excised from 1.5% agarose gel prepared in 1× TAE buffer with sterile gel slicer and purified using Clean Genei Gel Extraction kit (Bangalore Genei, India). The purified fragment was cloned in to pGEMT-Easy vector according to supplier's instructions. The ligation-reaction mixture was transformed into fresh competent cells of *Escherichia coli* JM109 strain. And recombinants were selected based on blue white screening. Plasmid DNA from recombinants was isolated using modified alkaline lysis method of Brinboim and Dolly¹⁹. The size of the insert DNA and ligation was confirmed by restriction digestion with *Eco*R1 restriction enzyme. Sequence analysis of the insert was done in an ABI 3730 automated DNA sequencer.

Sequence homology searches were performed using BLAST algorithm at <http://www.ncbi.nlm.nih.gov> of the National Center for Biotechnology Information (NCBI), with the program BLASTN.

Designing of SCAR Primer and Validation

A specific primer pair was designed based on the sequence of the cloned RAPD product, using the program PRIMER-BLAST, for 'Assam' type specific amplification identified by the RAPD marker. While designing the primer care was taken to avoid possible secondary structure or primer dimer generation and false priming, and to match melting temperature so as to achieve better primer annealing and amplification. The SCAR primers pair was used for PCR amplifications of genomic DNA from all the *Camellia* accessions.

Thermal cycling conditions for amplification using SCAR primers were optimized as: 94°C for 5 min; 45 cycles at 92°C for 30 sec, 62°C for 30 sec. and 72°C for 2 min; and a final extension at 72°C for 5 min. Amplified products were resolved by electrophoresis in 1.5% agarose gel.

Results

Identification of RAPD Marker for *C. assamica*

The procedure of DNA extraction yielded 400-600 ng of good quality DNA per 100 mg of tissue. An absorbance (A_{260}/A_{280}) ratio of 1.6-1.8 indicated insignificant levels of contaminating proteins and polysaccharides. Of the 20 primers screened, 11 primers produced distinct, reproducible amplification profile for all the accessions studied. Among these, primer OPA-10 consistently amplified a single, intense band of approx 811 bp for both the *C. assamica* cultivars TV 5 and TV 6. This particular 811 bp product was absent in rest of the 8 *Camellia* accessions (Fig. 1). This band OPA 10₈₁₁, was selected as putative *C. assamica*-specific marker.

Cloning and Sequencing of RAPD Marker OPA 10₈₁₁

OPA 10₈₁₁ was cloned in to pGEMT-Easy vector and white recombinant colonies were selected as transformants with 810 base pairs DNA insert (Fig. 2). Plasmid DNA from recombinants was isolated and digested with *Eco*R1. Restriction digestion analysis

revealed two bands, one corresponding to the size of intact wild type plasmid DNA (3015 bp) and the other to the size of specific DNA insert (811 bp) on 1.5% w/v agarose gel. This confirmed the presence of insert in the vector (Fig. 3). The excised fragment on sequencing revealed that first 10 nucleotides of the sequence, matched exactly with the corresponding RAPD primers used. Sequence homology searches

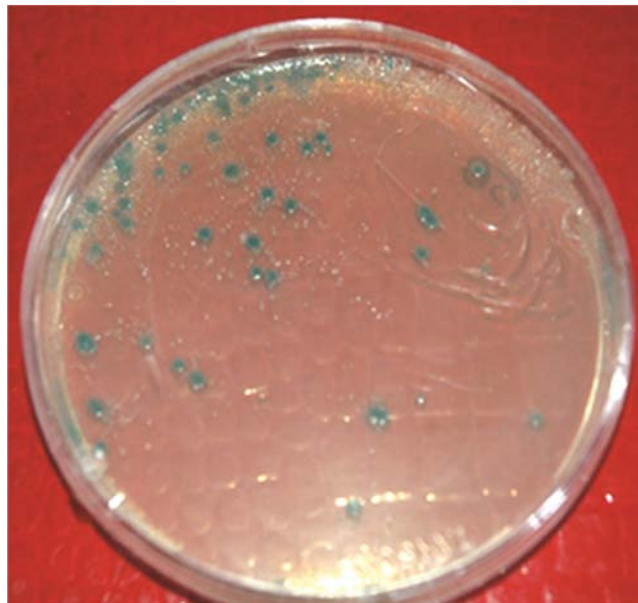


Fig. 2—*E. coli* JM109 strain transformed with plasmid having insert of 811 bp (white colonies represent recombinant and blue colonies represent non-recombinant individuals).

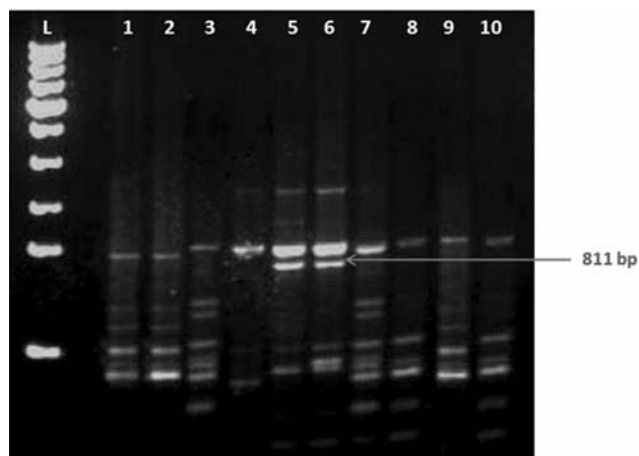


Fig. 1—RAPD fingerprints of *Camellia* germplasms generated by primer OPA-10: Lane M, 500 bp DNA marker (Genei, Bangalore); Lane 1, TV1 (Assam China hybrid); Lane 2: TV7 (China hybrid); Lane 3, TV16e (Assam hybrid); Lane 4, TV17 (Assam hybrid); Lane 5, TV5 (Assam type); Lane 6, TV6 (Assam type); Lane 7: TV18 (Cambod type) Lane 8, TV19 (Cambod type); Lane 9: TV29 (Cambod triploid); & Lane 10, TV30 (Cambod hybrid).

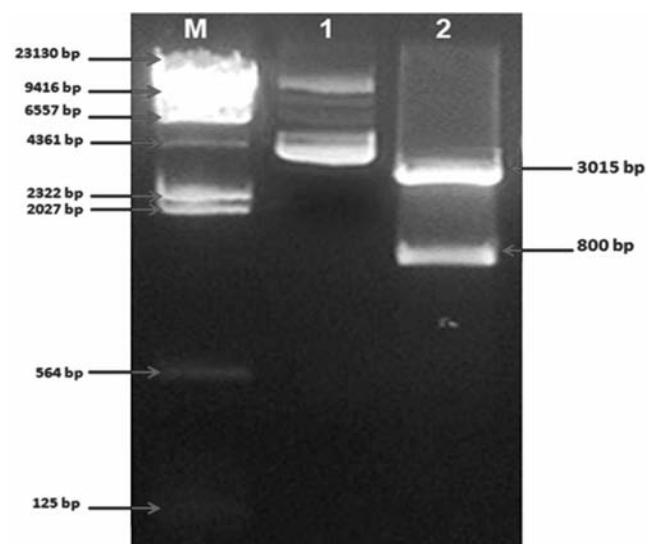


Fig. 3—Electrophoretic pattern of plasmid DNA digested with *Eco*RI: Lane M, Lambda DNA/*Eco*RI+*Hind*III; Lane 1, Undigested recombinant plasmid DNA; & Lane 2, Plasmid DNA digested with *Eco*RI.

Table 1—‘Assam’ type specific SCAR primer pair derived from cloned RAPD sequence

RAPD primer	SCAR primer	Sequence (5′-3′)	Length (bp)	T _m (°C)	GC%	Annealing temp* (°C)	Expected size of SCAR marker (bp)
OPA-10	Forward (F1)	AGTGAATTCGCGGCCGCCTG	20	60.38	65.00	62	640
	Reverse (R1)	CGATCGGTGCGGGCCTTTGT	20	60.04	65.00		

were further carried out using BLASTN program. It revealed that the sequence has homology with known plant nucleotide sequences at different sequence similarity levels. The length of the sequence obtained was 811bp with 50.68% G+C content (A=216; T=184; G=213; C=198).

Designing of SCAR Primers

In order to increase the specificity and reproducibility of RAPD marker, a pair of specific SCAR markers designated as F1 (forward) and R1 (reverse) of 20 base pairs in length were designed based on the sequence of marker OPA 10₈₁₁ (Table 1).

Validation of SCAR Primers with *Camellia* species

The designed SCAR primer pair was used to amplify genomic DNA from the all the ten *Camellia* accessions. A single, distinct, bright and easily identifiable band of 640 bp was amplified in both the *C. assamica* (TV5 & TV6) accessions, while no non-specific band was found in any other *Camellia* accessions studied (Fig. 4). Reduction of the annealing temperatures did not generate any fragment other than the SCAR. So the SCAR primer pair (F1 & R1) designed in this study proved to be a specific marker for ‘Assam’ type tea.

Discussion

RAPD analysis can reveal high degree of polymorphism without requiring prior DNA sequence information of the species and it is easy to perform. In the present study, RAPD marker specific to ‘Assam’ type tea accessions was identified. Twenty (decamer) RAPD primers were screened and among them 11 primers revealed polymorphism for different types of *Camellia* accessions studied. It was found that primer OPA-10 amplified an 811 bp band (OPA 10₈₁₁), which was unique for *C. assamica* (Assam type) accessions. Similar study has also been done in *Pisum sativum*, wherein a 750 bp fragment specific to Nord cultivar was resolved²⁰. Further, 80 RAPD primers were screened to develop DNA based marker for identification of *Phyllanthus emblica* and a putative marker of (1.1 kb) specific for *P. emblica* was

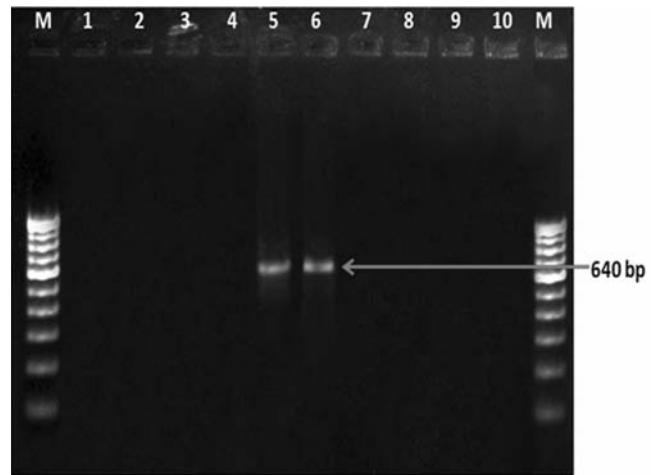


Fig. 4—Electrophoretic pattern of genomic DNA of *C. assamica* with the designed SCAR primers: Lane M, 100 bp DNA marker (Genei, Bangalore); Lane 1, TV1 (Assam China hybrid); Lane 2: TV7 (China hybrid); Lane 3, TV16 (Assam hybrid); Lane 4, TV17 (Assam hybrid); Lane 5, TV5 (Assam type); Lane 6, TV6 (Assam type); Lane 7: TV18 (Cambod type) Lane 8, TV19 (Cambod type); Lane 9: TV29 (Cambod triploid); & Lane 10, TV30 (Cambod hybrid).

identified by RAPD primer OPA-16¹³. In order to increase the specificity of the RAPD markers, these unique primers have to be converted into SCAR markers. SCAR markers have been developed in many crops like bentgrass²¹, *Sinocalycanthus chinensis*¹⁷, *P. emblica*¹³ and wheat²². Further, SCARs linked to disease resistance genes have been developed in crops like wheat²³ and soybean¹⁴.

Sequencing of PCR products using terminal primers would usually generate incomplete sequence data at both ends²⁴. So the cloning is preferred over direct sequencing in order to acquire complete and analyzed sequence data. In view of these, the identified RAPD marker OPA 10₈₁₁ was cloned in to pGEMT-Easy vector and sequenced. The sequence of this marker was utilized to generate specific, longer primer. Based on the sequences of RAPD marker OPA 10₈₁₁, a pair of SCARs (F1 & R1) was generated that amplified approx 640 bp in only *C. assamica* accessions, making the marker specific and dependable compared to RAPD markers. Similar kind

of results were also reported in *Pueraria tuberosa*, where 20 decamer RAPD primers of Operon Technologies (USA) were screened and a putative 600 bp marker specific to *P. tuberosa* was identified by primer OPA-08. The specific marker was cloned into pDRIVE cloning vector, sequenced and designed into SCAR primers, which produced a single band of 320 bp in only *P. tuberosa*²⁵.

The development of SCAR marker is a credible and convenient solution for identifying *C. assamica* other than morphological method, especially during early stage. More recently, breeders have been using interspecific hybridization to combine important traits. Hybridizations of different species can be useful for incorporating traits, such as, disease or drought resistance, into new cultivars, making it critically important to identify the hybrids. The longer length (20 bp) and higher annealing temperature (65°C) of SCAR primers make the reaction more specific, reproducible and stable. The designed pair of SCAR markers has potential for use as a diagnostic tool for cultivar identification.

Acknowledgment

The authors thankfully acknowledge the financial assistance in terms of studentship provided by the Department of Biotechnology (DBT), Government of India, New Delhi.

References

- Sealy J R, A revision of the genus *Camellia* (The Royal Horticultural Society, London) 1958.
- Rajkumar S, Karthigeyan S, Sud R K, Rajkumar R, Muraleedaran N *et al*, Genetic diversity of Indian tea (*Camellia sinensis* (L.) Kuntze) germplasm detected using morphological characteristics, *J Cell Plant Sci*, 1 (2010) 13-22.
- Wight W, Tea classification revised, *Curr Sci*, 31 (1962) 298-299.
- Lin Y S, Tasi Y J, Tsav J S & Lin J K, Factors affecting the levels of tea polyphenols and caffeine in tea leaves, *J Agric Food Chem*, 51 (2003) 1864-1873.
- Hertog M G L, Hollman P C H, Katan M B & Kromhout D, Intake of potentially anticarcinogenic flavonoids and their determinants in adults in the Netherlands, *Nutr Cancer*, 20 (1993) 21-29.
- Pan X, Niu G & Liu N, Microwave-assisted extraction of tea polyphenols and tea caffeine from green tea leaves, *Chem Eng Process*, 42 (2003) 129-133.
- Mondal T K & Chand P K, Detection of genetic variation among micropropagated tea [*C. sinensis* (L.) O. Kuntze] by RAPD analysis, *In Vitro Cell Dev Biol*, 38 (2002) 296-299.
- Munthali M, Ford-Lloyd B V & Newbury H J, The random amplification of polymorphic DNA for fingerprinting plants, *PCR Methods Appl*, 1 (1992) 274-276.
- William J G, Kubelik A R, Livak K J, Rafalsky J A & Tingey S V, DNA polymorphism amplified by arbitrary primers are useful as genetic markers, *Nucleic Acids Res*, 18 (1990) 6531-6335.
- Da Cunha Galvao L M & Lages-Silva E, Randomly amplified polymorphic DNA (RAPD)—A useful tool for genomic characterization of different organisms, in *Molecular biomethods handbook*, 2nd edn, edited by J M Walker and R Rapley (Humana Press, Totowa, NJ, USA) 2008, 133-147.
- Lee G P, Lee C H & Kim C S, Molecular markers derived from RAPD, SCAR and the conserved 18S rDNA sequences for classification and identification in *Pyrus pyrifolia* and *P. communis*, *Theor Appl Genet*, 108 (2004) 1487-1491.
- Rugienius R, Sikesnianas T, Stanys V, Gelvonaukiene D & Bendokas V, Use of RAPD and SCAR markers for identification of strawberry genotypes carrying red stele (*Phytophthora fragariae*) resistance gene *RPfl*, *Agron Res*, 4 (2006) 335-339.
- Warude D, Chavan P, Joshi K & Patwardhan B, Development and application of RAPD-SCAR marker for identification of *Phyllanthus emblica* Linn., *Biol Pharm Bull*, 29 (2006) 2313-2316.
- Zheng C, Chang R, Qiu L, Chen P, Wu X *et al*, Identification and characterization of a RAPD/SCAR marker linked to a resistance gene for soybean mosaic virus in soybean, *Euphytica*, 132 (2003) 199-210.
- Mc Dermott J M, Brandle U, Dutly F, Haemmerli U A, Keller S *et al*, Genetic variation in powdery mildew of barley: Development of RAPD, SCAR, and VNTR markers, *Phytopathology*, 84 (1994) 1316-1321.
- Paran I & Michelmore R W, Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce, *Theor Appl Genet*, 85 (1993) 985-999.
- Ye Q, Qiu Y-X, Quo Y-Q, Chen J-X, Yang S-Z *et al*, Species-specific SCAR markers for authentication of *Sinocalycanthus chinensis*, *J Zhejiang Univ Sci B*, 7 (2006) 868-872.
- Dellaporta S L, Wood J & Hicks J B, Maize DNA minipreps, *Maize Genet Crop Newslett*, 57 (1983) 26-29.
- Birnboim H P & Dolly J, A rapid alkaline extraction procedure for screening recombinant plasmid DNA, *Nucleic Acids Res*, 7 (1979) 1513-1523.
- Koveza O V, Kokaeva Z G, Gostimskii S A, Petrova T V & Osipova E S, [Production of a SCAR marker in pea *Pisum sativum* L. using RAPD analysis] [Article in Russian], *Genetika*, 37 (2001) 574-576.
- Scheef E A, Casler M D & Jung G, Development of species-specific SCAR markers in Bent grass, *Crop Sci*, 43 (2003) 345-349.
- Hernandez P, Martin A & Dorado G, Development of SCARs by direct sequencing of RAPD products: A practical tool for the introgression and marker-assisted selection of wheat, *Mol Breed*, 5 (1999) 245-253.
- Gold J, Harder D, Townley-Smith F, Aung T & Procinier J, Development of a molecular marker for rust resistance genes *Sr39* and *Lr35* in wheat breeding lines, *Electronic J Biotechnol*, 2 (1999). [Doi: 10.2225/vol2-issue1-fulltext-1]
- Singh M, Chaudhary K, Singal H R, Magill C W & Boora K S, Identification and characterization of RAPD and SCAR markers linked to anthracnose resistance gene in sorghum [*Sorghum bicolor* (L.) Moench], *Euphytica*, 149 (2006) 179-187.
- Devaiah K & Venkatasubramanian P, Development of SCAR marker for authentication of *Pueraria tuberosa* (Roxb. ex Willd.) DC., *Curr Sci*, 94 (2008) 1306-1309.