BIHAREAN BIOLOGIST 5 (1): pp.17-24
Article No.: 111101

http://biologie-oradea.xhost.ro/BihBiol/index.html

Genetic relationship among indigenous coffee species from India using RAPD, ISSR and SRAP markers

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Received: 05. December 2010 / Accepted: 17. January 2011 / Available online: 02. February 2011

Abstract. Three molecular marker systems, RAPD (random amplified polymorphic DNA), ISSR (inter-simple sequence repeat) and SRAP (sequence related amplified polymorphism), were employed for identification and genetic relationship between five indigenous coffee species from India and cultivated species *Coffea canephora*. A total of 304, 140 and 234 bands were detected by 22 RAPD, 12 ISSR and 20 SRAP primer combinations, among which 95%, 91.3% and 96.1% bands were polymorphic respectively. The average PIC of SRAP primers (0.82) was higher than RAPD primers (0.78) but lesser than that of ISSR primers (0.83) where as the average RP of SRAP primers (8.3) is higher than ISSR primers (8.2) but less than RAPD primers (10.3). Some of the RAPD and SRAP primers were able to distinguish all the indigenous coffee species independently. The genetic similarity among the species ranged from 0.34 to 0.75 using RAPD, 0.30 to 0.69 using ISSR and 0.23 to 0.63 using SRAP marker systems. Based on the marker analysis, all the six species were clustered in to two major groups. The results demonstrated that these three marker systems could be useful for identification and genetic diversity analysis of indigenous coffee species and provide an important input in to conservation biology.

Key words: Indian coffee species, Genetic relationships, Inter simple sequence repeats (ISSR), Random amplified polymorphic DNA (RAPD); Sequence related amplified polymorphism (SRAP).

Introduction

The genus *Coffea* L. belongs to the tribe Coffeeae of the family Rubiaceae and contains more than 100 species (Davis et al. 2006). Majority of the coffee species occurs naturally in Africa, Madagascar and Mascarenes predominantly restricted to humid evergreen forest but some species are found in seasonally dry deciduous forest and /or bush land (Maurin et al. 2007). The importance of coffee as an agricultural commodity relies mainly upon two varieties *Coffea arabica* and *C. canephora* contributing about 65% and 35% of total production respectively. In spite of the commercial and social importance of the genus, the genetic relationship between majorities of the coffee species has not been extensively studied and the taxonomic status of the genus within the tribe coffeeae is poorly understood. Recently, Davis et al (2006) published a detail annotated taxonomic conspectus of the genus *Coffea* and included five indigenous coffee species from India, of which *C. bengalensis*, *C. travancorensis*, *C. wighitiana* are placed under the genus *Psilanthus* and other two species such as C. *khasiana* and *C. jenkinsii* are placed under the genus *Nostolachma* both under Coffeeae. Based on the analysis of internal transcribed spacers (ITS) of nuclear DNA, Lashermes et al. (1997), observed limited sequence divergence between *Psilanthus* and *Coffea* and concluded that *Psilanthus* should not be recognised as a separate genus from *Coffea*. Based on the comparative sequence analysis of plastid DNA, Cros et al. (1998) concurred with Lashermes et al. (1997) about the close relationship between *Coffea* and *Psilanthus* although their tree topology shows an unresolved relationship between two species of *Psilanthus* (*P.mannii* and *P.ebracteolatus*) studied by them and *Coffea*. However, both these studies did not include representative of closely related genera for comparison. Further studies by Andreasen et al. (1999), Andreasen and Bremer (2003) and Davies et al. (2007) showed that *Psilanthus* is nested within *Coffea* and stressed the importance of species level studies to resolve the phylogenetic relationship. Maurin et al. (2007) observed that the separation of *Coffea* and *Psilanthus* based on the morphological features is not convincing and suggested to include molecular data from other species of *Psilanthus* to establish the relationship between these two genera. Based on the morphological features, *Nostolachma* is placed under Coffeeae (Davis et al. 2007) although molecular data from additional species may prove to be useful. Several indigenous coffee species were reported from India by various workers (Narasimhaswamy & Vishweswra 1963, Sivarajan et al. 1992). Wild plant species form an important link between the ecological adaptation and evolutionary process and contain many useful genes for genetic improvement of the cultivated species (Dulloo & Owadally 1991). For example, the indigenous coffee species such as *C. bengalensis*, *C. travancorensis* and *C. wightiana* have nil or only traces of caffeine and therefore could be useful for developing coffee varieties with less caffeine using traditional breeding or biotechnological tools (Sreenath et al. 1992). In coffee, fertile intergeneric hybrids, involving *C. arabica* and *Psilanthus ebracteolus* (Couturon et al. 1998) were recovered and this has strengthened the possibility of utilising wild coffee species in genetic improvement. However, most of the indigenous coffee species from India face extinction and ex situ germplasm conservation is threatened by destruction of natural habitat. Conservation management of rare and endangered taxa often need quick decision without adequate ecological data or information on their genetic variability (Gaston & Kunin, 1997). Molecular markers such as RAPD, ISSR and SRAP analysis have proved useful in estimating the genetic diversity in a variety of plant species such as mulberry (Vijayan et al. 2006), Pongamia (Sahoo et al. 2010), radish (Wang et al. 2008), Celosia (Feng et al. 2009) including coffee (Lashermes et al. 1993., Orozco-Castilo et al. 1996., Anthony et al. 2001) and thereby facilitating the conservation strategies. In this context, information on diversity and genetic relationship and among wild species is essential for the identification of potential species for conservation. There is no report on the genetic relationship among the indigenous coffee species from India. In the present study, we report the genetic relationship

within the five indigenous coffee species from India using RAPD, ISSR and SRAP molecular markers. The main objectives of the study are to generate a molecular database and identification tag for each species that may be useful for conservation and systematic studies.

Materials and Methods

Plant Materials

Leaf material was collected from 10 individuals of five indigenous coffee species viz. *C. bengalensis*, *C. travancorensis*, *C.wightiana*, *C. kasiana* and *C. jenkinsii* from wild forest and maintained at the germplasm plot of Central Coffee Research Institute, Chikmagalur, Karnataka and Regional Coffee Research Station Thandigudi, Tamil nadu. Mature seeds were also collected and grown in 10 in earthen pots under net house conditions. The leaf materials were used for DNA isolation.

DNA extraction

Genomic DNA was extracted from fresh young leaves using a modified CTAB method. About 200 mg of leaf tissue was ground to fine powder in liquid nitrogen, then transferred to 30 ml tube containing 5 ml preheated extraction buffer (2% CTAB (w/v), 100mM Tris-HCL (pH 8.0), 25mM EDTA, 2M Nacl, 0.1 % beta- mercaptoethanol). The tubes were incubated at 60^0 C for 1 hour with occasional shaking. After incubation, the tubes were cooled to the room temperature and centrifuged at 6000 rpm for 20 min. The supernatant was transferred into a new tube and extracted twice with equal vol. of chloroform-isoamyl alcohol (24:1). The supernatant was transferred to 2ml tubes, precipitated with 0.7 vol. of isopropanol at room temperature for 30 min., and then centrifuged at 8000 rpm for 20 min at 4°C. The pellet formed after centrifugation was washed with 75% (v/v) ethanol for 10 min and dissolved in 60 µl of Tris-EDTA (1-10mM). The concentration of DNA was measured using 0.8% agarose gel stained with ethidium bromide as well as by UV spectro photometer at 260 nm and 280 nm. The resuspended DNA was then diluted in sterile distilled water to 10ng/ µl concentration for use in amplification reactions.

RAPD, ISSR, SRAP analysis

A total of 65 decamer primers from Operon Technologies (Almaeda, California), 20 ISSR primers from University of British Columbia and 60 SRAP primer combinations (8 forward primers and 14 reverse primers) synthesized by Sigma, India were initially screened to determine the suitability of each primer for the study. Primers were selected for further analysis based on their ability to detect clear and distinct polymorphic amplification products within the species of *Coffea*. Twenty-two RAPD primers, ten ISSR primers and twenty SRAP primer combinations that had a high level of polymorphism and the best readability were used for PCR amplification (Table 1). PCR was carried out in a palm cycler (Corbett Research).

RAPD PCR were carried out in 20 µl volume containing 20 ng template DNA, 200 µM each of deoxynucleotide triphosphate, 0.3 µM of primer, 2.0 mM $MgCl_2$, 1x reaction buffer (10 mM Tris HCL pH 8.8, 50mM KCL0.08% Nonidet P40) and 1.5 U *Taq* DNA polymerase. The RAPD amplification conditions were: 4 min initial denaturation at 95^o C; 35 cycles consisting of 1 min denaturation at 94^oC; 1.15 min primer annealing at 38^oC and 2 min extension at 72^oC and a final 10 min extension at 72^oC.

ISSR PCR were conducted in 20 µl reaction mixture containing 1x reaction buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl 0.08% Nonidet P40), 30 ng DNA, 200 µM dNTP mixture, 2.5 mM $MgCl_2$, 3 µM ISSR primer,0.2 µl of formamide and 1.0 U *Taq* DNA polymerase. The ISSR amplification conditions were: 3 min initial denaturation at 95^o C; 30 cycles consisting of 1 min denaturation at 94^oC; 1.30 min primer annealing at 55^oC and 2 min extension at 72^oC and a final 10 min extension at 72 ^oC.

SRAP analysis was performed by adapting the procedure described by Li and Quiros (2001) with minor modifications: 20 µl reaction mixture containing 1x reaction buffer (75mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2$ SO_4, 0.01% Tween 20), 30 ng template DNA, 200 µM dNTP mixture, 2.5 mM $MgCl_2$, 3 µM each of forward and reverse primer, and 1.0 U *Taq* DNA polymerase. The SRAP amplification program was 4 min initial denaturation at 96^oC; 5 cycles consisting of 1 min denaturation at 94^oC, 1.15 min primer annealing at 35^oC; and 2 min extension at 72^oC followed by 30 cycles consisting of 1 min denaturation at 94^o C, 1.15 min primer annealing at 50^o C; and 2 min extension at 72^oC; and a final extension of 15 min at 72^oC.

The RAPD PCR products were analysed by electrophoresis on 1.5% (w/w) agarose gels where as PCR products of both ISSR and SRAP were run on 2.0% (w/w) agarose gels containing 0.5 µg ethidium bromide/ml in 1X TAE buffer and then visualized and photographed using the UV-transilluminator (SYNGENE) and documented using the Gene Snap software program. All the three PCR were repeated at least twice to confirm the reproducibility of each PCR band.

Data Analysis

The RAPD, ISSR and SRAP amplified bands were scored for presence (1) or absence (0). The total number of bands, distribution of bands across all species, polymorphic bands, species-specific bands and average number bands per primer were calculated. The value of each primer was assessed using two indices; PIC, which is the same as diversity index (Botstein et al. 1980; Milbourne et al. 1997) and Resolving power (Rp) (Prevost & Wilkinson, 1999). PIC or DI was estimated as PIC= $(1-p^2_i)/n$, where n is the

Table 1. Primer sequence used for RAPD, ISSR and SRAP analysis of Indian coffee species.

RAPD		ISSR		SRAP	
Primer code	Sequence of the primer (5′ – 3′)	Primer code	Sequence of the primer (5′ – 3′)	Forward primer (5′ – 3′)	Reverse primer (5′ – 3′)
OPAD-15	TTTGCCCCGT	UBC- 810	$(GA)_8T$	Me1TGAGTCCAAACCGGATA	Em2 GACTGCGTACGAATTTGC
OPAG-15	CCCACACGCA	UBC - 811	$(GA)_8C$	Me2 TGAGTCCAAACCGGAGC	Em3 GACTGCGTACGAATTGAC
OPAB-03	TGGCGCACAC	UBC- 826	$(AC)_8C$	Me3 TGAGTCCAAACCGGAAT	Em4 GACTGCGTACGAATTTGA
OPAN-05	GGGTGCAGTT	UBC- 834	$(AG)_8YT$	Me4 TGAGTCCAAACCGGACC	Em5 GACTGCGTACGAATTAAC
OPAS-09	TGGAGTCCCC	UBC-835	$(AG)_8YC$	Me6 TGAGTCCAAACCGGACA	Em6 GACTGCGTACGAATTGCA
OPAR-10	TGGGGCTGTC	UBC-836	$(AG)_8YA$	Me9 TGAGTCCAAACCGGAGG	Em7 GACTGCGTACGAATTCAA
OPAJ-14	ACCGATGCTG	UBC-840	$(GA)_8YT$	Me10 TGAGTCCAAACCGGAAA	Em9 GACTGCGTACGAATTCAG
OPAL-12	CCCAGGCTAC	UBC-841	$(GA)_8YC$	Me11 TGAGTCCAAACCGGAAC	Em10 GACTGCGTACGAATTCAT
OPAP-20	CCCGGATACA	UBC-842	$(GA)_8YG$		Em11 GACTGCGTACGAATTCTA
OPAX-06	AGGCATCGTG	UBC-855	$(AC)_8YT$		Em12 GACTGCGTACGAATTCTC
OPBH-04	ACCTGCCAAC	UBC-880	GGAGAGGAGAGGAGA		Em13 GACTGCGTACGAATTCTG
OPBG-18	TGGCGCTGGT	UBC-881	GGGTGGGGTGGGGTG		Em14 GACTGCGTACGAATTCTT
OPI-08	TTTGCCCGGT				Em15 GACTGCGTACGAATTGAT
OPL-20	TGGTGGACCA				Em16 GACTGCGTACGAATTGTC
OPC-11	AAAGCTGCGG				
OPL12	GGGCGGTACT				
OPN-02	ACCAGGGGCA				
OPU-06	ACCTTTGCGG				
OPS-10	ACCGTTCCAG				
OPU17	ACCTGGGGAG				
OPBF-09	ACCCAGGTTG				
BOT-54	TTTGGGTGCG				

number of band positions analyzed in all the species, p_i is the frequency of the ith banding pattern. The resolving power of a primer is Rp = □ I_b where I_b (band informativeness) takes the value of 1- [2x (0.5-p)] and p is the ratio of six species sharing the band. A pair wise similarity matrix was constructed using the Dice similarity coefficient (Sneath & Sokal 1973). The relationship between the species was displayed as a dendrogram constructed using NTSYS –PC 2.10e software (Rohlf 1995) based on Unweighted Pair Group Method using Arithmatic averages (UPGMA). Statistical support of the clusters was assessed by means of 1000-bootstrap replicates.

Results

RAPD polymorphism and species identification

Sixty-five RAPD primers were initially screened against five indigenous coffee species and one cultivated species, (*C. canephora*) of which 22 primers are found highly polymorphic and produce clear amplification pattern. These 22 primers could produce 304 distinct reproducible bands with an average of 13.8 per primer (Table 2). The size of the amplified products ranged from 200 to 2500 bp. Of the total 304 bands obtained, 289 were polymorphic, with an average 13.1 polymorphic fragments per primer. Percent of polymorphism ranged from 62.5% to a maximum of 100% with an average of 95% polymorphism. Out of 22 primers, 13 primers showed 100% polymorphism. The resolving power of 22 RAPD primers tested ranged from 4.0 (OPL-20) to 20.3 (OPAG-15) with an average of 10.3. Similarly, the polymorphism information content (PIC) or the genetic diversity of 22 RAPD primers ranged from 0.22 (OPBG-18) to 0.93 (OPL-20) with average of 0.78.

Some of the polymorphic RAPD primers such as OPC-11, OPL-12 and OPN-02 could discriminate all the species independently (Fig. 1a). Six species generated 119 unique fragments out of which five Indian species generated 90 unique fragments. Among the Indian species, maximum number of unique fragments were generated by *C. jenkinsii* (26) followed by *C. bengalensis* (24) and least number of unique fragments in *C. wightiana* (6), which could be used as unique fingerprinting tools. Some of these unique RAPD fragments could be used as diagnostic markers for discriminating the species (Table 3). The genetic similarity, derived from the data of the RAPD marker analysis, varied from 0.20 between *C. wightiana* and *C. khasiana* to 0.60 between *C. travancorensis* and *C. wightiana* (Table 4). Although majority of the Indian species showed similar level of genetic similarity with *C. canehora*, *C. khasiana* and *C. travancorensis* showed highest genetic similarity based on the RAPD marker analysis.

The dendrogram based on RAPD data was constructed by UPGMA analysis that grouped the five indigenous coffee species and *C. canephora* into two major clusters (Fig. 2a). Among the two major clusters, one major cluster had only one species (*C. jenkinsii*) and the other major cluster was divided into two minor clusters. First minor cluster was represented by *C. canephora* and *C. khasiana* and the second minor cluster consisted of *C. travancorensis* and *C. wightiana* and *C. bengalensis*. All the Indian species share more than 20% similarity among themselves. The dendrogram represents the close distances among the species occurring in adjacent tips of the classification according to numerical taxonomy (Sneath and Sokal 1973).

ISSR polymorphism and species identification

Out of twenty ISSR primers initially screened, 12 primers were found to be polymorphic and produced clear and reproducible amplification pattern. These 12 primers could produce 140 distinct reproducible bands across the six species with an average of 11.7 per primer (Table 2). The size of the amplified products ranged from 100 to 2900 bp. Of the total 140 amplified bands, 137 were polymorphic, with an average of 11.4 polymorphic fragments per primer. All the ISSR primers except UBC-881 and UBC-855 showed 100% polymorphism. The primers UBC-881 and UBC-855 showed 8.3% and 87.5% of polymorphism respectively. The average polymorphism percentage recorded with ISSR primers is 91.3. The resolving power of 12 ISSR primers tested ranged from 5.0 (UBC- 826) to 22.7 (UBC-881) with an average of 8.2. Similarly, the polymorphism information content (PIC) of 12 ISSR primers ranged from 0.07 (UBC- 881) to 0.99 (UBC-835 and UBC-840) with average of 0.83.

None of the ISSR polymorphic primers could discriminate all the species independently. However, the primer UBC-880 can differentiate all the species except *C. travancorensis* by amplifying species diagnostic fragments (Table 3). Twelve ISSR primers generated 63 unique fragments in five species except *C. travancorensis* where no species-specific fragments could be generated. The four Indian species generated 39 unique fragments of which maximum numbers of unique fragments were generated by *C. jenkinsii* (14) and the least number of unique fragments by *C. bengalensis* (2). Of the total 39 unique fragments, 28 unique fragments which are prominently amplified are given in Table 3 and these fragments could be used as species diagnostic markers. The genetic similarity derived from the data of the ISSR marker analysis varied from 0.18 between *C. canephora* and *C. bengalensis* to 0.53 between *C. travancorensis* and *C. wightiana* (Table 5). Among the Indian species, *C. jenkinsii* showed maximum genetic similarity with *C. canehora*, based on the ISSR marker analysis.

The dendrogram based on ISSR data was constructed by UPGMA analysis and grouped the six species into two major clusters (Fig. 2b). The first major cluster was represented by *C. canephora* and *C. jenkinsii* and the second major cluster divided into two minor clusters of which the first minor cluster consisted of *C. travancorensis C. wightiana* and *C. bengalensis* and the second minor cluster included *C. khasiana*. All the Indian species share more than 20% similarity among themselves.

SRAP polymorphism and species identification

Sixty SRAP primer combinations were screened against five indigenous coffee species along with the cultivated species, *C. canephora* of which 20 primer combinations are found highly polymorphic and produce clear amplification pattern. These 20 primers could produce 234 distinct scorable bands among six species. The number of amplified fragments ranged from seven (Me2-Em3) to sixteen (Me2-Em12, Me4-Em11) with an average of 11.7 per primer combinations (Table 2). The size of the amplified products ranged from 50 to 2500 bp. Of the total 234 amplified bands, 225 were polymorphic, with an average 11.3 polymorphic fragments per primer combinations. Percent of polymorphism ranged from 81.8% to a maximum of 100% with an average of 96.1%

polymorphism. Out of 20 tested primer combinations, 12 primer combinations showed 100% polymorphism. The resolving power (RP) of 20 SRAP primer combinations ranged from 3.7 (Me10-Em14) to 13.0 (Me2-Em10) with an average of 8.3. Similarly, the polymorphism information content (PIC) or the genetic diversity of 20 SRAP primer combinations ranged from 0.73 (Me3-Em4) to 0.90 (Me1-Em12, Me11-Em11) with average of 0.82.

Table 2. Polymorphism obtained by RAPD, ISSR and SRAP analysis in coffee species

Primer/ primer combinations	Total bands	Size range (bp)	Number of bands in each species	No. of Polymorphic bands	Percentage of polymorphism	RP	PIC
RAPD							
OPAD-15	11	500-1800	2-6 (2.5)	11	100	5.0	0.82
OPAG-15	22	300-2000	7-11 (10.1)	21	95.5	20.3	0.72
OPAB-03	10	250-1000	3-7 (3.8)	10	100	7.6	0.81
OPAN-05	13	200-2200	1-7 (4.7)	13	100	9.3	0.82
OPAS-09	19	350-2000	5-11 (7.2)	18	94.7	14.3	0.82
OPAR-10	24	300-2000	3-11 (7.5)	24	100	15.0	0.88
OPAJ-14	15	250-1400	3-9 (4.8)	15	100	9.6	0.87
OPAL-12	18	350-1600	3-7 (5.3)	17	94.4	10.7	0.87
OPAP-20	16	250-1500	2-8 (4.3)	16	100	8.6	0.89
OPAX-06	12	250-2000	3-6 (5.0)	10	83.3	10	0.72
OPBH-04	16	250-1400	1-8 (4.8)	16	100	9.6	0.88
OPBG-18	8	300-2100	6-8 (6.7)	6	62.5	13.3	0.22
OPI-08	10	350-1900	1-4 (2.8)	10	100	4.66	0.90
OPL-20	8	400-1500	1-5 (2.0)	8	100	4.0	0.93
OPC-11	12	300-2300	3-6 (4.2)	11	91.6	8.3	0.81
OPL12	13	200-2000	6-9 (7.0)	9	69.2	14	0.59
OPN-02	17	250-1900	3-8 (6.5)	16	94.1	13	0.78
OPU-06	17	200-1900	5-12 (9.1)	15	88.2	18	0.60
OPS-10	11	400-1200	3-6 (4.3)	11	100	8.6	0.79
OPU17	9	400-1700	3-4 (3.3)	9	100	6.6	0.82
OPBF-09	12	300-2000	1-7 (4.5)	12	100	9	0.79
BOT-54	11	250-2500	1-9 (3.2)	11	100	6.3	0.87
Total	304		66-154 (113.5)	289		225.8	17.2
Average	13.8		3-7 (5.2)	13.1	95.0	10.3	0.78
ISSR							
UBC- 810	11	150-2000	1-8 (3.5)	11	100	7.0	0.88
UBC - 811	10	300-1800	2-6 (3.2)	10	100	6.3	0.89
UBC- 826	9	300-2200	1-4 (2.5)	9	100	5.0	0.90
UBC- 834	10	250-1900	2-5 (3.0)	10	100	6.0	0.86
UBC-835	12	200-2500	1-7 (2.8)	12	100	5.7	0.99
UBC-836	11	150-2000	1-6 (3.0)	11	100	6.0	0.90
UBC-840	14	200-1900	2-6 (3.7)	14	100	7.3	0.99
UBC-841	10	200-2000	1-6 (2.8)	10	100	5.6	0.89
UBC-842	12	200-1450	2-5 (4.2)	12	100	8.3	0.85
UBC-855	16	300-2900	2-12 (5.3)	14	87.5	10.7	0.82
UBC-880	13	150-2000	2-6 (4.2)	13	100	8.3	0.87
UBC-881	12	100-2500	11-12 (11.3)	11	8.3	22.7	0.07
Total	140		28-83 (49.5)	137		98.9	9.91
Average	11.7		2.3 -6.9 (4.1)	11.4	91.3	8.2	0.83
SRAP							
Me1-Em2	15	150-2000	5-6 (5.5)	15	100	11.0	0.81
Me1-Em4	12	250-1500	3-5 (4.3)	11	91.7	8.7	0.82
Me1-Em6	8	750-1800	1-4 (2.5)	7	87.5	5.0	0.83
Me1-Em12	13	150-1200	3-6 (4.3)	13	100	8.7	0.90
Me2-Em3	7	750-1800	1-3 (2.7)	6	85.7	5.3	0.78
Me2-Em4	10	150-2500	1-5 (3.5)	10	100	7.0	0.85
Me2-Em10	16	50-2500	4-9 (6.5)	15	97.3	13.0	0.78
Me2-Em12	16	50-1000	3-7 (4.8)	16	100	9.6	0.86
Me3-Em3	8	500-2000	1-4 (2.8)	8	100	5.7	0.82
Me3-Em4	12	100=1500	3-8 (5.2)	11	91.7	10.3	0.73
Me3-Em9	10	75-1000	2-6 (4.3)	10	100	8.7	0.76
Me3-Em11	10	75-600	1-5 (2.7)	10	100	5.3	0.89
Me3-Em12	15	100-1500	4-9 (6.2)	14	93.3	12.3	0.76
Me4-Em11	16	100 - 2000	1-9 (6.2)	15	93.8	12.3	0.78
Me6-Em5	11	75-1300	2-9 (3.7)	11	100	7.3	0.86
Me9-Em15	13	100-2000	2-8 (4.5)	13	100	9.0	0.85
Me10-Em14	8	100-1500	1-3 (1.8)	8	100	3.7	0.84
Me10-Em13	11	100-1000	3-6 (4.3)	9	81.8	8.7	0.75
Me11-Em11	12	75-1100	3-6 (3.3)	12	100	6.7	0.90
Me11-Em16	11	100-1000	2-5 (3.7)	11	100	7.3	0.88
Total	234		46-123 (76.6)	225		165.6	16.5
Average	11.7		2.3 – 6.2(3.8)	11.3	96.1	8.3	0.82

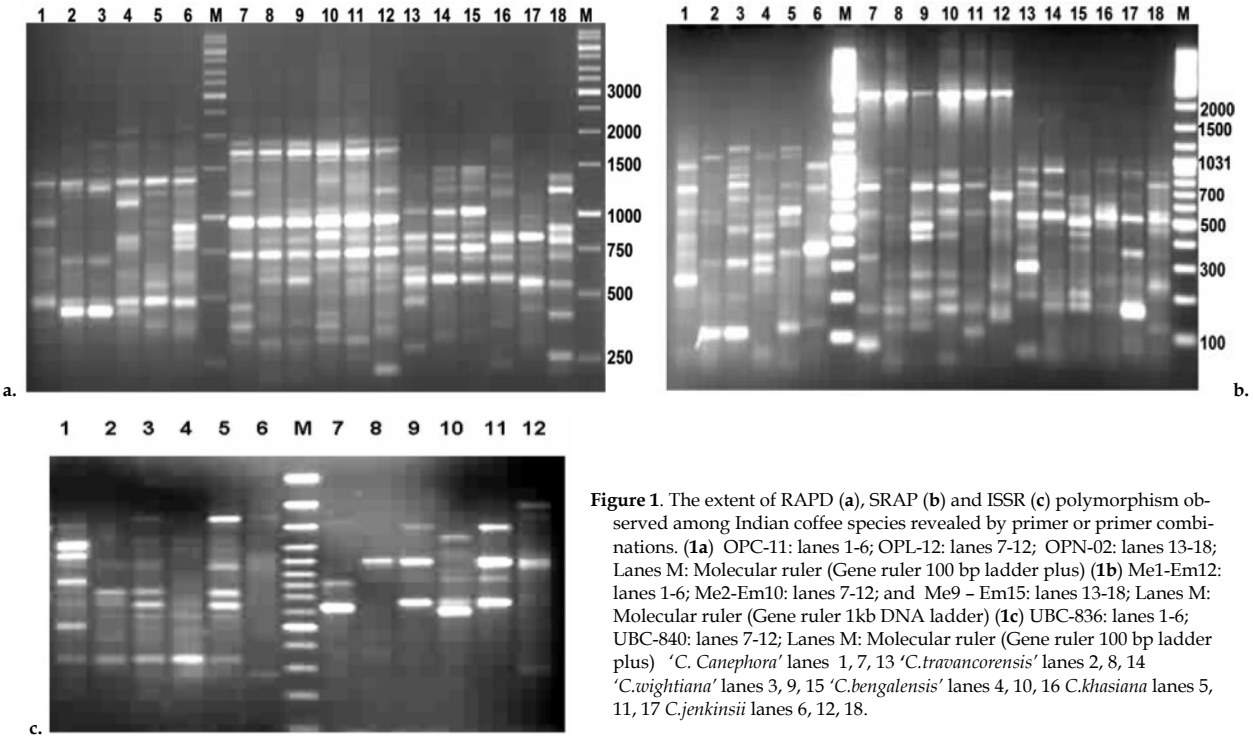

Figure 1. The extent of RAPD (**a**), SRAP (**b**) and ISSR (**c**) polymorphism observed among Indian coffee species revealed by primer or primer combinations. (**1a**) OPC-11: lanes 1-6; OPL-12: lanes 7-12; OPN-02: lanes 13-18; Lanes M: Molecular ruler (Gene ruler 100 bp ladder plus) (**1b**) Me1-Em12: lanes 1-6; Me2-Em10: lanes 7-12; and Me9 – Em15: lanes 13-18; Lanes M: Molecular ruler (Gene ruler 1kb DNA ladder) (**1c**) UBC-836: lanes 1-6; UBC-840: lanes 7-12; Lanes M: Molecular ruler (Gene ruler 100 bp ladder plus) '*C. Canephora*' lanes 1, 7, 13 '*C.travancorensis*' lanes 2, 8, 14 '*C.wightiana*' lanes 3, 9, 15 '*C.bengalensis*' lanes 4, 10, 16 *C.khasiana* lanes 5, 11, 17 *C.jenkinsii* lanes 6, 12, 18.

Table 3. Species- diagnostic RAPD, ISSR and SRAP markers in Indian coffee species.

Species	RAPD marker	ISSR marker	SRAP marker
C. bengalensis	OPAB-03 - 1000 OPAG-15 – 450 OPAN-05 – 950, 2200 OPAR-10 – 400 OPAJ-14 -1200 OPAX-06 – 250, 1450 OPBH-04 – 900 OPBH-04 – 900 OPC-11 – 1200 OPU17- 750, 1700 OPBF-09 - 1950	UBC880 – 200, 270 UBC840 – 600, 1300	Me1-Em2-740 Me1-Em6-400 Me1-Em12-300 Me2-Em4-400 Me11-Em11- 280, 410
C. travancorensis	OPAB-03 – 250 OPAN-05 – 200, 750 OPL-20 – 800 BOT-54- 450		Me1-Em4-250 Me1-Em6-650 Me3-Em4- 350
C. whitiana	OPC-11 – 1400 OPU-06 - 1200	UBC810- 700 UBC841 – 1900, 2000 UBC842 – 1200 UBC855 – 1700 UBC880 - 2000	Me1-Em2-700 Me1-Em6-1500 Me1-Em12-800 Me2-Em3-850 Me2-Em10-480 Me2-Em12-450 Me10-Em14-220, 1300 Me11-Em11- 300
C. khasiana	OPAG-15 – 350 OPAS-09 – 350 OPAJ-14 – 300, 550 OPAL-12 – 400 OPAX-06 – 300 OPU17 – 850 OPBF-09 – 700, 800 BOT-54 - 350	UBC811- 600 UBC841 - 900 UBC842 – 230 UBC855 – 950, 1030 UBC880 - 610	Me2-Em4-300, 480,530 Me2-Em10-110 Me2-Em12-180,350 Me3-Em4- 320 Me3-Em12-1250, 1400 Me9-Em15-250, 800 Me10-Em14- 470 Me11-Em11- 220
C .jenkinsii	OPAD-15 – 700, 1000 OPAX-06 – 1400 OPI-08 – 700, 1000 OPN-02 – 400, 900, 1300 OPU-06 – 200 OPBF-09 – 1200 BOT-54 - 1000	UBC834- 500, 950 UBC840 – 280, 2000 UBC841- 930 UBC842- 300, 1150 UBC855 – 300, 1500, 2000, 2900 UBC880 – 900, 1050	Me1-Em2-150, 550 Me1-Em6-260 Me2-Em10-700 Me3-Em3-500 Me3-Em4- 250,375, 490, 550, 1100 Me3-Em4- 900 Me6-Em5-350 Me9-Em15-830 Me10-Em14- 470

Table 4. Similarity co-efficient of Indian coffee species using RAPD marker (DICE).

Species	*C. canephora*	*C. travancorensis*	*C. wightiana*	*C. bengalensis*	*C. khasiana*	*C. jenkinsii*
C. canephora	1.00					
C. travancorensis	0.31	1.00				
C. wightiana	0.28	0.60	1.00			
C. bengalensis	0.30	0.32	0.32	1.00		
C. khasiana	0.31	0.25	0.20	0.28	1.00	
C. jenkinsii	0.27	0.23	0.21	0.28	0.26	1.00

Table 5. Similarity co-efficient of Indian coffee species using ISSR marker (DICE).

Species	*C. canephora*	*C. travancorensis*	*C. wightiana*	*C. bengalensis*	*C. khasiana*	*C. jenkinsii*
C. canephora	1.00					
C. travancorensis	0.26	1.00				
C. wightiana	0.31	0.53	1.00			
C. bengalensis	0.18	0.45	0.34	1.00		
C. khasiana	0.25	0.26	0.21	0.27	1.00	
C. jenkinsii	0.36	0.22	0.25	0.21	0.20	1.00

Table 6. Similarity co-efficient of Indian coffee species using SRAP marker (DICE).

Species	*C. canephora*	*C. travancorensis*	*C. wightiana*	*C. bengalensis*	*C. khasiana*	*C. jenkinsii*
C. canephora	1.00					
C. travancorensis	0.26	1.00				
C. wightiana	0.17	0.31	1.00			
C. bengalensis	0.28	0.46	0.31	1.00		
C. khasiana	0.22	0.35	0.19	0.34	1.00	
C. jenkinsii	0.23	0.23	0.13	0.22	0.25	1.00

Table 7. Similarity co-efficient of Indian coffee species using RAPD, ISSR & SRAP markers (DICE)

Species	*C. canephora*	*C. travancorensis*	*C. wightiana*	*C. bengalensis*	*C. khasiana*	*C. jenkinsii*
C. canephora	1.00					
C. travancorensis	0.28	1.00				
C. wightiana	0.24	0.48	1.00			
C. bengalensis	0.27	0.39	0.32	1.00		
C. khasiana	0.27	0.29	0.20	0.30	1.00	
C. jenkinsii	0.28	0.23	0.19	0.25	0.24	1.00

It was observed that few SRAP polymorphic primer combinations such as Me1-Em12, Me2-Em10 and Me9 – Em15 could identify all the species independently (Fig. 1b). Twenty SRAP primer combinations generated 107 unique fragments in six species out of which five Indian species generated 79 unique fragments. Among the Indian species, maximum number of unique fragments were generated by *C. wightiana* (26) followed by *C. jenkinsiis* (21) and least number of unique fragments in C. travancorensis (5) which could be used as unique fingerprinting tools. Of the total 79 unique fragments, 45 fragments which are clear and robust are selected for species diagnostic markers (Table 3). The genetic similarity, derived from the data of the SRAP marker analysis, varied from 0.17 between *C. wightiana* and *C. canephora* to 0.46 between *C. travancorensis* and *C. bengalensis* (Table 6). Among the Indian species, *C. bengalensis* showed maximum similarity with *C. canephora*, where as *C. wightiana* showed least genetic similarity based on the SRAP marker analysis.

The UPGMA clustering algorithm from SRAP analysis grouped the five indigenous coffee species and *C. canephora* into two major clusters (Fig. 2c). The first major cluster was represented by *C. canephora* and *C. jenkinsii*. The second major cluster divided into two minor clusters of which the first minor cluster consisted of *C. travancorensis, C. bengalensis* and *C. khasiana* and the second minor cluster included *C. wightiana*. A strict consensus tree based on the RAPD, ISSR and SRAP data were constructed (Fig. 2d). This dendrogram shows maximum similarity to ISSR based dendrogram. Using all the three markers maximum genetic similarities were obtained between *C. travancorensis* and *C. wightiana* where as lowest genetic similarity was obtained between *C. khasiana and C. jenkinsii* (Table 7).

Discussion

Analysis of crop genetic diversity is very important for breeding and conservation programs, and molecular markers offer an approach to unveil the genetic diversity among different species and cultivars based on nucleic acid polymorphisms. In this study, three markers, RAPD, ISSR and SRAP were simultaneously used to investigate the genetic diversity among five indigenous coffee species from India. The results showed that all the three markers were suitable to genetic diversity analysis in coffee by amplifying several species specific diagnostic markers. Species specific RAPD markers were generated in a variety of tree plant species such as *Eucalyptus* (Grattapaglia et al. 1992), *Picea* (Khasa & Dancik 1996), and Acacia (Nanda et al. 2004). Similarly, ISSR primers were also used as species specific diagnostic markers in Eucalyptus (Balasaravanan et al. 2006) and Oak (Carvalho et al. 2009). In the present study, species-diagnostic

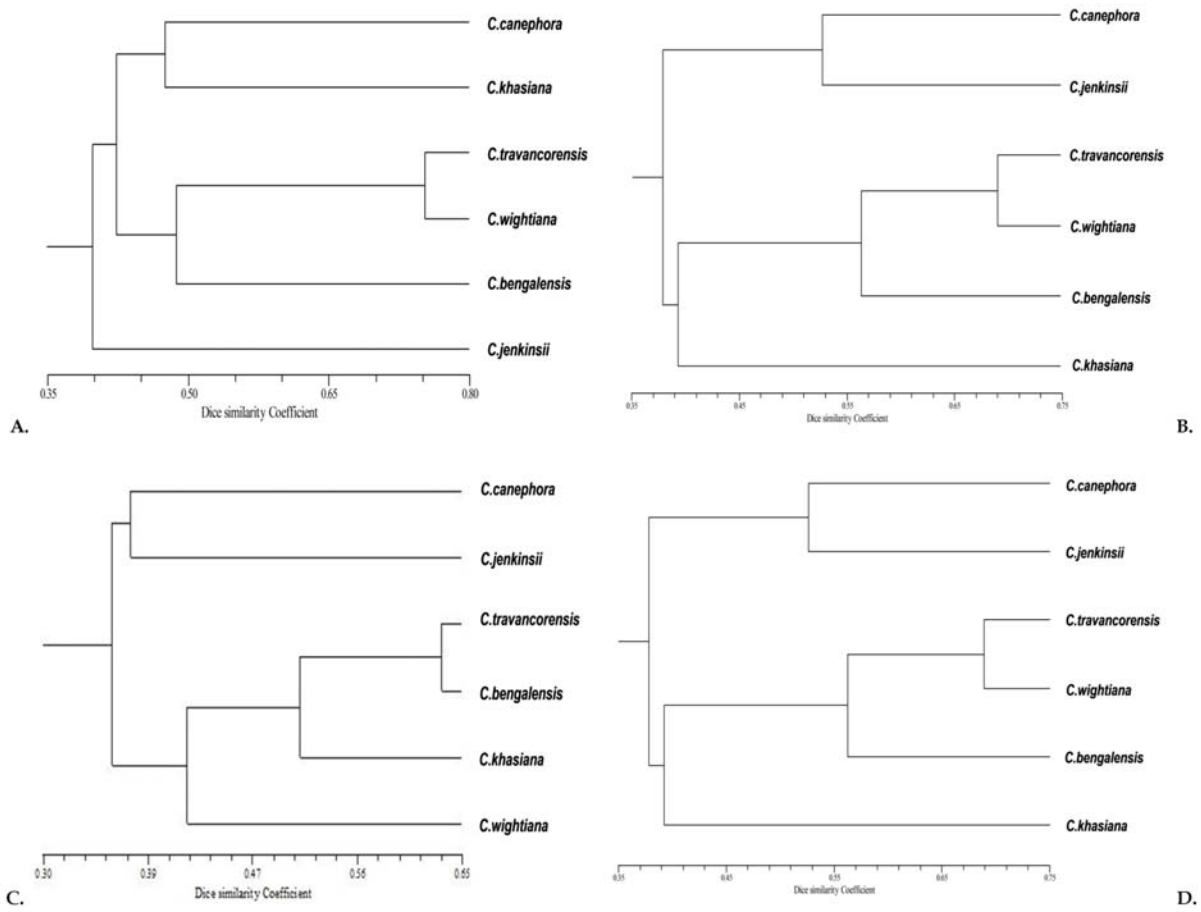

Figure 2. Dendrogram generated using unweighted pair group method with arithmetic average (UPGMA) analysis., showing relationships among different coffee species from India, using RAPD (**A**), ISSR (**B**) , SRAP (**C**) and a strict consensus based on RAPD, ISSR and SRAP data (**D**).

ISSR markers were identified in all the indigenous coffee species from India except *C. travancorensis*. Similar problems were experienced while developing species-specific ISSR markers in *Eucalyptus* (Balasaravanan et al. 2006). This could be due to the occurrence of high divergence among the particular species population and / or due to the low number of ISSR primers used in this study.

Both genetic factors and selection pressure influence the genetic diversity (Sun & Lin 2003) and therefore it is not surprising to find polymorphism among different indigenous coffee species. The percentage of polymorphic bands detected by SRAP primer combinations (96.1%) was higher compared to RAPD (95%), ISSR (91.3%) although the number of polymorphic bands detected by RAPD primer (13.1) was higher than both ISSR primer (11.4) and SRAP primer combinations (11.3). However the number of species diagnostic markers generated by RAPD and SRAP is much higher compared to ISSR marker. This indicated that both SRAP and RAPD markers are efficient in identifying the Indian species, although all the three markers are efficient in genetic diversity analysis of coffee.

The average PIC of SRAP primers (0.82) was higher than RAPD primers (0.78) but lesser than that of ISSR primers (0.83) where as the average RP of SRAP primers (8.3) is higher than ISSR primers (8.2) but less that RAPD primers (10.3). The difference in PIC and RP values among three different markers is expected because these markers differ in their operational principle and each marker targets a different region of the genome. This is evident from the UPGMA clustering of the indigenous coffee species as well as *C. canephora* using RAPD, ISR and SRAP markers. While both species, such as *C. travencorensis* and *C. wightiana* from southern peninsular India, showed maximum similarity between themselves (Tables 3, 4) and were placed close to each other in the dendrogram (Fig. 3a,b) using RAPD and ISSR markers. However, using SRAP marker, *C. travancorensis* shared maximum similarities with *C. bengalensis* (Table 5) and was placed closely in the dendrogram (Fig. 3c). Further, the genetic similarities observed between *C. canephora* and the indigenous coffee species was different using RAPD, ISSR and SRAP markers. While *C. khasiana* and *C. jenkinsii* showed maximum similarities with *C. canephora* using RAPD and ISSR markers, *C. bengalensis* showed maximum similarity with *C. canephora* using SRAP marker. Since RAPD marker system generally detects the neutral genetic variation and ISSR markers target the region within the microsatellite repeats, SRAP markers preferentially detect polymorphism in coding sequences which are usually conserved among closely related cultivars and species with low mutation rate. Therefore simultaneous use of different types of molecular markers may be useful in generating all – sided information.

In conclusion, this study provides the information on genetic relatedness not only among different indigenous coffee species from India but also provides information on their

relatedness with *C. canephora*, which is the only diploid cultivated species in the genus *Coffea*. An understanding of the level and partitioning of genetic variation within the species would provide an important input in designing appropriate breeding exercise and conservation strategies.

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