Research Article

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Inheritance of RAPD and ISSR marker in inter-specific coffee hybrids (*coffea congensis* froehner x *coffea canephora* pierre) and their derivatives: implications for genetic improvement

Deepanshu Kumar[1], Bharatha Nandhini[1], Surya Prakash Rao[2], Karthiga Sivamani[1], Thilaga Sethuraman[1] and Ganesh Doss[1*]

[1]*Department of Plant Biotechnology, School of Biotechnology, Madurai Kamaraj University, Palkalai Nagar 625 021, Madurai, Tamil Nadu, India*
[2]*Division of Plant Breeding and Genetics, Central Coffee Research Institute, Coffee Research Station Post 577 117, Chikmagalur District, Karnataka, India*

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Abstract

The C x R coffee hybrid (a cross between *Coffea congensis* and *Coffea canephora*) represents a significant development in coffee cultivation in India, addressing challenges like plant size optimization for high-density planting and enhancement of beverage quality. However, maintaining genetic purity in this hybrid is challenging due to genetic segregation caused by cross-pollination with other diploid species or within the hybrid itself. This highlights the need for effective tools like DNA marker-assisted selection (MAS) to improve the genetic stability and performance of this hybrid. In this study, two dominant DNA markers (RAPD and ISSR) were analyzed for their utility in coffee genetic improvement. Out of 24 RAPD primers and 15 ISSR primers tested, only 3 RAPD and 5 ISSR primers were effective in discriminating the parental species.These selective RAPD and ISSR primers produced nine and twenty threepolymorphic bands with size range of 400 bp – 1200 bp for RAPD and 100 bp – 2000 bp for ISSR, respectively. Species specific RAPD and ISSR markers were found in female (*Coffea congensis*) and male (*Coffea canephora*) parent of the hybrid. Some of these markers were successfully inherited by F1 hybrids and backcross progenies.The suitability of the primers for MAS was evaluated based on the parameters such as Polymorphic information content (PIC), effective multiplex ratio (EMR), marker index (MI) and resolving power (RP). The selected RAPD and ISSR markers enable effective discrimination between the parental species and their hybrids. This is crucial for ensuring the genetic purity of seeds and plants.The ability to use MAS for maintaining seed purity and selecting superior hybrids can significantly enhance the productivity and quality of C x R coffee hybrids, benefiting the coffee industry.The importance of these markers in genetic improvement of this hybrid is discussed.

Keywords: *Coffea congensis*, *Coffea canephora*, inter-specific hybrid; C x R Coffee hybrid, DNA markers, genetic improvement

Introduction

Coffea canephora Pierre (2n=2x=22), popularly known as 'Robusta coffee' is one of the important species cultivated in the world, contributing 30% of coffee production in the international market (Orozco-Castillo *et al.,* 1994; Ruas *et al.,* 2003; ICO 2018). *C. canephora* is the most suitable *Coffea* species for cultivation due to its durable resistance to major pest and diseases (Prakash *et al.,* 2005; Campuzano-Duque and Blair, 2022).

*Corresponding author: dossganesh@mkuniversity.ac.in

However, this species produces inferior quality of coffee bean as compared to *C. arabica* (2n=4x=44) which contributes the remaining 70% of world coffee production (ICO, 2018; Prakash *et al.,* 2005; Campuzano-Duque and Blair, 2022; Lashermes *et al.,* 1996; Berthaud and Charrier, 1988; Carvalho *et al.,* 1988). Therefore, genetic improvement of *C. canephora* for enhancement of coffee quality is a research priority in many coffee producing countries (Anthony *et al.,* 2002; Akpertey *et al.,* 2022). *C. congensis* is native to Africa and predominantly occurs along the basin of river Congo. This species received the attention of the researchers as it is the only one among the diploid species of *Coffea* that produces superior-quality coffee beans (Steiger *et al.,*2002; Leroy *et al.,* 2006). This species is highly compatible for inter-crossing with *C. canephora* and become one of the potential genetic resources for improving the coffee quality in *C. canephora* (Davis and Rakotonasolo, 2009; Bertrand *et al.,* 2023). Therefore, inter specific hybridization between *C. congensis* and *C. canephora* was employed in India to incorporate better beverage quality in *C. canephora* followed by a number of backcrossing with either of the parents to stabilize beverage quality and yield (Jamsheed *et al.*, 1996). Systematic breeding in India led to the development of Congensis × Robusta hybrid, popularly known as C × R hybrid. (Jamsheed *et al.*, 1998).

C × R hybrid is cultivated as mixed plantation in India with *Coffee canephora* (male parent of this hybrid) as an efficient pollen donor for improving the productivity. Under field condition, this hybrid is distinguishable morphologically from both the parents with an intermediate bush size with drooping behavior of primary branches (Anonymous, 1988). However, upon reaching maturity, this hybrid tends to alter its vegetative growth and resemble the male parent. This hybrid shows wide range of phenotypic variations due to influence of different agro-climatic condition, leading to practical difficulties for the farmers and researchers to distinguish C x R hybrid from *Coffee canephora* (Jamsheed *et al.*, 1996; Jamsheed *et al.*, 1998). A number of works on the utility of two dominant DNA markers such as RAPD (Orozco-Castillo*et al.,* 1994; Lashermes *et al.,* 1996; Agwanda *et al.,*1997; Aga *et al.,* 2003; Maluf *et al.,* 2005; Ramadiana *et al.,*2021; Wahibah *et al.,* 2023; Paulo *et al.,*2003; Lin *et al.,* 2019; Panaligan *et al.*, 2020; Amruthakumar *et al.,* 2023) are reported for analyzing the genetic diversity of *Coffea* species and several coffee hybrids. Although most of the reports on DNA markers are relating to genetic diversity of *Coffea*, only a few works could demonstrate the application of DNA marker in molecular assisted selection (MAS)-based breeding in coffee. For example, ISSR marker was used for determination of cultivated coffee varieties and inter-specific hybrids *Coffea* (Lin *et al.,* 2019; Panaligan *et al.*, 2020). A panel of SNPs for identification of twelve arabica coffee cultivars has been reported (Amruthakumar *et al.,* 2023; Lin *et al.,* 2016; Tran *et al.,* 2018). Our study revealed a few unique ISSR markers to distinct the parental species of a commercially important inter-specific hybrid of coffee and demonstrated that these markers transmit into F1 hybrid and its advanced progenies. Utilization of these markers in genetic improvement of this hybrid and other possible applications are discussed.

Materials and Methods

Plant materials

Young leaves of *C. congensis*, *C. canephora*, *C. congensis* × *C. canephora* (F1 hybrid) and backcross progeny of C × R to *C. canephora* were collected from the germplasm block of Central Coffee Research Institute, Chikmagalur District, Karnataka State, India. Derivation of this hybrid through conventional breeding (Fig.1) and their characteristic

features are described (Table 1). Whole leaves were washed with sterile distilled water, placed inpolypropylene bags and stored at − 70°C (Sanyo, Japan) until further processing. Frozen leaf samples were used for DNA extraction later.

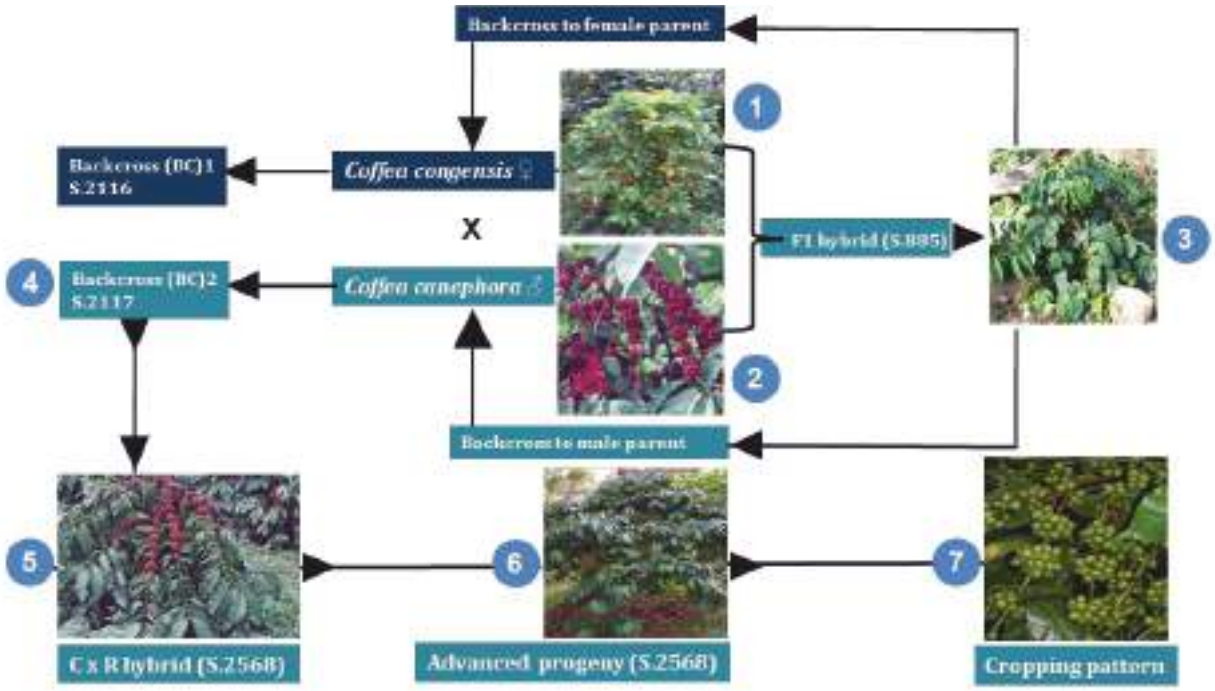


Figure 1. Derivation of C x R coffee hybrid: 1, *coffea congensis*- female parent with small bush size and with poor cropping 2, *coffea canephora* – male parent with larger bush size and with higher crop 3, C x R F1 hybrid with intermediate bush size and with broader leaves 5, C xR hybrid derived from the backcross of F1 with *C. canephora* (accession S.2568) 6 &7, old plants of advanced progeny showing larger bush size similar to *C. canephora* with high cropping.

Table I. Parental information and characteristics

Parent/ hybrids	Origin and characteristic features
C. congensis (♀)	Native to Africa and predominantly occurring along the basin of river Congo, highly tolerant against continuous water logging, compact bush with relatively smaller leaves than *C. canephora*, internodes are shorter as compared to other diploid species of *Coffea,* poor yielder but with higher aroma quality than *C. canephora*
C. canephora(♂)	Exotic collection, introduced into India from Java, highly resistant to major pests and diseases of coffee and suitable for cultivation under low elevation, highly productive.
C. congensis x *C. canephora* (F1 hybrid)	An interspecific hybrid involving *Coffeacongensis* and *Coffeacanephora*, compact plant stature, suitable for high density planting to increase the productivity, superior in flavour quality as compared to *C. canephora*.
Backcross of F1 (♀) to *C. canephora* (♂)	Compact plant stature with high yield potential, suitable for high density planting to increase the productivity, superior in flavour quality

Isolation of genomic DNA

About 0.5 g of frozen leaf tissue of coffee was ground into fine powder inliquid N_2 using sterile/chilled mortar and pestle. The powder samples were added into 1 ml of extraction buffer (100 mM Tris-pH 8.0, 1.4M NaCl, 20mM EDTA- pH 8.0, 2% CTAB, 0.3% β mercaptoethanol and 1% PVP) in a centrifuge tube and incubated at 60°C for 60 min in heating block (Thermo Fisher Scientific, Mumbai, India). Samples were allowed to attain normal temperature and equal volumes of (24:1) chloroform and isoamyl alcohol (HiMedia Laboratories, Mumbai, India) were added and gently mixed to form emulsion. Samples were centrifuged at 12,000 rpm for 10 min (Kubota, Japan). After centrifugation, supernatant was gently recovered without disturbing the pellet. The supernatant was washed once again with chloroform and isoamyl alcohol and subjected to next round of centrifugation at 12,000 rpm for 10 min. Clear supernatant was recovered and to it 2/3 volume of the isopropanol was added (HiMedia Laboratories, Mumbai, India) and incubated at −80°C (Cryo Scientific Systems Private Limited, Chennai, India) for 60 min. Samples were once again centrifuged at 12,000 rpm for 12 min and the supernatant was carefully recovered without disturbing DNA pellet and washed with 20 µl of 70% ethanol before centrifugation at 12,000 rpm for 5 min. Ethanol was removed by micropipette and the final DNA pellet was vacuum dried for 15 min. DNA pellet was re-suspended in 50 µl of 0.1 × TE buffer-pH8 (10 mMTris, 1 mM EDTA) and stored at −20°C. The integrity of the DNA was verified in 0.8% agarose gel (Sigma Aldrich, Mumbai, India) and the quality of DNA ascertained. Quantification of DNA was carried out using UV-Vis spectrophotometer (Bio Rad, India) purity of DNA was determined by OD_{260}/OD_{280} ratio.

Screening of RAPD and ISSR primers

Template DNA of *Coffea congensis* was initially used for screening with 24 RAPD 15 ISSR primers procured from Sigma Aldrich, India (Table 2 and 3). The selected RAPD and ISSR primers were procured based on the previously published work (Ruas *et al.,*2003; Mishra *et al.*,2011). These primers were diluted as per the manufacturer protocol and stored at -20°C (Blue Star, India). The polymerase chain reaction (PCR) mixture and conditions for amplification of genomic DNA using RAPD and ISSR primers are given Table 4. PCR amplification was performed with a Thermal Cycler (Himedia, India) and PCR products were run in 1.5% agarose gel (Hi Media, India) and ISSR fingerprints were analysed for number of scorable and polymorphic bands and documented.

Table 2. RAPD and primer sequences

Primer	Sequence	Primer	Sequence
OPAP 20	5'- CCCGGATACA -3'	OPN 02	5'- ACCAGGGGCA-3'
OPI 08	5'- TTTGCCCGGT -3'	OPAD 07	5'- CCCTACTGGT-3'
OPAS 09	5'- TGGAGTCCCC -3'	OPAD 07	5'- CCCTACTGGT-3'
OPAB 03	5'- TGGCGCACAC-3'	OPAN 05	5'- GGGTGCAGTT-3'
OPAD 15	5'- TTTGCCCCGT- 3'	OPL 12	5'- GGGCGGTACT-3'
OPO 09	5'- TCCCACGCAA-3'	OPBH 04	5'- CCCGGATACA-3'
OPAR 10	5'- TGGGGCTGTC-3'	OPAX 06	5'- AGGCATCGTG-3'
OPO 14	5'- AGCATGGCTC-3'	OPI 07	5'- CAGCGACAAG-3'
OPAG 15	5'- CCCACACGCA-3'	OPS 10	5'- ACCGTTCCAG-3'
OPBF 09	5'- ACCCAGGTTG-3'	OPC 11	5'- AAAGCTGCGG-3'
OPK 05	5'- TCTGTCGAGG-3'	OPP 20	5'- GACCCTAGTC-3'
OPAL 12	5'- CCCAGGCTAC-3'	OPF 14	5'- TGCTGCAGGT-3'

Table 3. Details of ISSR primers used in this study

ISSR Primers	Sequence (5' – 3')	Repeats	Primer Name	Reference
SSR 01	GAGAGAGAGAGAGAGAT	(GA) 8T	UBC-810*	[Ruas *et al.* 2003; Panaligan, Baltazarand Alejandro 2020 Amruthakumar *et al.* 2023; Mishra, Nishani and Jayarama 2011]
SSR 02	GAGAGAGAGAGAGAGAC	(GA) 8C	UBC-811*	,,
SSR03	ACACACACACACACACC	(AC) 8C	UBC-826*	,,
ISSR04	AGAGAGAGAGAGAGAGYT	(AG) 8 YT	UBC-834*	,,
ISSR05	AGAGAGAGAGAGAGAGYC	(AG) 8 YC	UBC-835*	,,
ISSR06	AGAGAGAGAGAGAGAGYA	AG) 8 YA	UBC-836 *	,,
ISSR07	GAGAGAGAGAGAGAGAYT	(AG) 8 YT	UBC-840*	,,
ISSR08	GAGAGAGAGAGAGAGAYC	(GA) 8 YC	UBC-841*	,,
SSR09	GAGAGAGAGAGAGAGAYG	(GA) 8 YG	UBC-842*	[Ruas *et al.* 2003; Amruthakumar *et al*. 2023; Mishra, Nishani and Jayarama 2011]
ISSR10	ACACACACACACACACYT	(GA) 8 YT	UBC-855*	[Ruas et al. 2003; Panaligan, Baltazarand Alejandro2020; Amruthakumar *et al.* 2023; Mishra, Nishani and Jayarama 2011]
ISSR11	GGAGAGGAGAGGAGA	(GGAGA) 3	UBC-880*	[Ruas *et al.* 2003; Amruthakumar *et al*. 2023; Mishra, Nishani and Jayarama 2011]
ISSR12	GGGTGGGGTGGGGTG		UBC-881*	,,
ISSR13	AGAGAGAGAGAGAGAGAG**	(AG) 9		,,
ISSR14	CTCTCTCTCTCTCTCTG**	(CT) 8 G		,,
ISSR15	GAGAGAGAGAGAGAGAGAT*	(GA) 9 T		,,

* Primers ISSR01 – ISSR12 and Primers ISSR13 – ISSR15 were originally synthesized by University of British Colomiba (UBC) and Life Technologies respectively.

Table 4. Composition of PCR and run conditions for amplification of RAPD and ISSR

PCR Mixture/Condition	RAPD	ISSR
Template DNA	30 ng	30 ng
10 x PCR Buffer	2.5 µl	3.0 µl
$MgCl_2$	1.6 µl (2.5 mM)	1.6 µl (3.0 mM)
dNTPs	0.5 µl (2.5 mM)	0.5 µl (10 mM)
Primers	(10 pmole/µl)	10 pmoles/µl
Taq DNA *Pol.*	0.3µl (3U)	0.3µl (5U)
BSA	--	0.3µl (10 mg/ml)
PVP	--	3.0 µl (10%)
Initial denaturation	94°C for 4 min	94°C for 3 min
Denaturation	95°C for 1 min	95°C for 1 min
Annealing temperature	37°C for 1 min (37 cyc)	55°C for 1 min 30 sec (55 cyc)
Extension	72°C for 2 min	72°C for 2 min
Final extension	72°C for 6 min	72°C for 10 min

Development of DNA fingerprints of parents and hybrids

Following the initial screening, three RAPD (OPAB3, OPAL12, OPAL15) and five ISSR (ISSR1, ISSR2, ISSR6, ISSR9, ISSR13) primers, were used further to develop DNA markers specific for *C. congensis* (female parent) and *Coffea canephora* (male parent). To determine the inheritance of ISSR markers to F1 hybrid (S.885) and its backcross progeny to *C. canephora* (S.2568) and advanced progeny (S.2568), parental samples were included for PCR amplification with F1 and backcross progeny. Fingerprints were carefully analyzed by comparing between parent and its derivatives. Reproducible and scorable nature of bands observed in the hybrid derivatives were compared with either of the parent and repeated the amplification at least five times for reproducibility. The ISSR marker inherited from parents to F1 hybrid and its backcross and advanced progenies were analyzed for marker inheritance and documented.

DNA Marker analysis

RAPD and ISSR marker analysis was done based on the assumption that each band represented the phenotype at a single biallelic locus. Consistent and scorable bands were selected for data generation. To detect the molecular weight of unique and species-specific DNA bands corresponding to *C. congensis* and *C. canephora* and their derivatives, fingerprints were photographed using Bio-Rad Gel Documentation system and each row of the gel was analyzed using EgyGene GelAnalyzer4 Program based on the guidelines (https://egygenes.blogspot.com). By comparing the banding pattern of female (*C. congensis*) and male (*C. canephora*) parent and their F1 and backcross progenies, species-specific bands were detected and molecular weight was determined. Binary data was used to calculate number of scored bands (NSB), number of polymorphic band (NPB), and percentage of polymorphic bands (PPB). To determine the efficiency of ISSR markers, four parameters such as polymorphic information content (PIC), effective multiplex ratio (EMR), marker index (MI) and resolving power (RP) were analyzed.

Polymorphic information content (PIC)

PIC value of each DNA band was calculated based on the formula $PIC_{i=}$ 2fi $(1\text{-}f_i)$ (Zhang *et al.,* 2021). Where PIC_i is the PIC of the band i, f_i is the frequency of the amplified fragment (band present) and $1\text{-}f_i$ is the frequency of non-amplified fragment (band absent). The frequency was calculated as the ratio between the number of amplified bands at each locus and the total number of progenies. The PIC of each primer was calculated using the average PIC value from all loci of each primer.

Marker index (MI)

Marker index (MI) was calculated to characterize the capability of each primer to detect polymorphic loci between the parents, hybrid and its backcross derivatives as demonstrated previously (Roldan *et al.,* 2000). MI=EMR × PIC; Where, EMR (effective multiplex ratio) = $n \times \beta$, where n is the average number of fragments amplified in each accession/genotype and β is estimated from the number of polymorphic loci (PB) and the number of non-polymorphic loci (MB). Therefore, β = PB/ (PB + MB) as described by Varshney *et al.,*(2007).

Resolving power (RP)

Resolving power of each primer was calculated based on the following formula (Varshney *et al.,* 2007). RP = ΣI_b, Where I_b represents the informative fragments. The I_b can be represented on a scale of 0/1 by the following formula; $I_b = 1 - [2 \times (0.5 - p_i)]$, Where p_i is the proportion of accessions containing the i^{th} band.

Inheritance of ISSR markers

Parental specific DNA marker corresponding to *C. congensis* (female parent) and *C. canephora* (male parent) were scored and their molecular weights were determined using EgyGene GelAnalyzer4 Program. Subsequently, these unique markers were analyzed critically for their inheritance into the hybrid and its backcross progenies.

Results

Screening of ISSR primers

A total of 25 RAPD and 15 ISSR primers were selected for screening based on the published reports (Ruas *et al.,* 2003; Prevost and Wilkinson *et al.,*1999). These primers were screened once again primarily to select most suitable oligonucleotides to ensure better reproducibility for molecular characterization of C x R hybrid and its progenies. The number of amplicons produced by each primer was varying from one to fourteen and size of the amplified bands ranged from 250 – 2800 bp for RAPD (Fig. II). For ISSR primers, three to nine bands were amplified with molecular weights ranging from 200 to 1300 bp (Fig. III). Based on the number of reproducible bands, three RAPD primers (OPAB-03, OPAL-12, OPAG-15) and five ISSR primers (ISSR-1, ISSR-2, ISSR-6, ISSR-9, ISSR 13) were selected for developing DNA markers for precise identification of C x R hybrid. Genomic DNA of selected parental samples (*C. congensis* and *C. canephora*) and their hybrid derivatives produced unique fingerprints upon the PCR amplification using the selective RAPD and ISSR primers.

Comparison of RAPD and ISSR markers

A total of 10 loci from RAPD and 26 loci from ISSR were obtained in C x R coffee hybrid and its derivatives. Most of the PCR products were in the size range of 250-2800 bp for RAPD and 200 bp-1300 bp for ISSR with mean value 3.3 and 8.8 amplicons per primer, respectively. From the above data, nine and twenty three loci of RAPD and ISSR were

Figure 2. Screening of RAPD primers: twenty-four RAPD primers showing the number of amplicons (a) and low and high molecular weight amplicons from the pooled genomic DNA of *coffea congensis* and *coffea canephora*

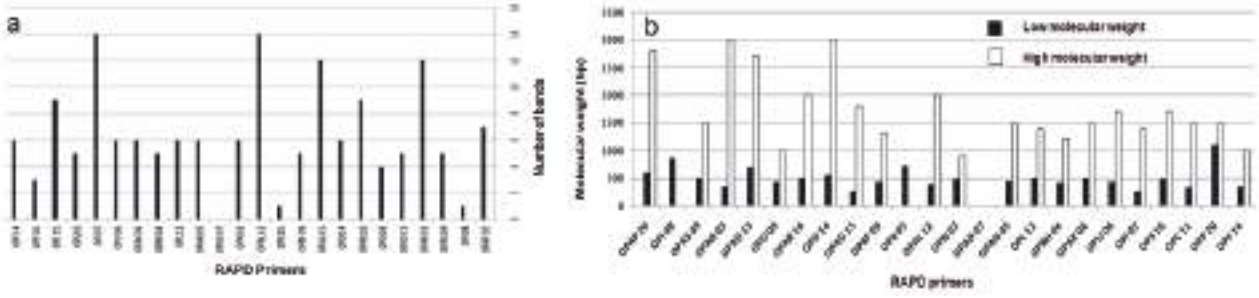
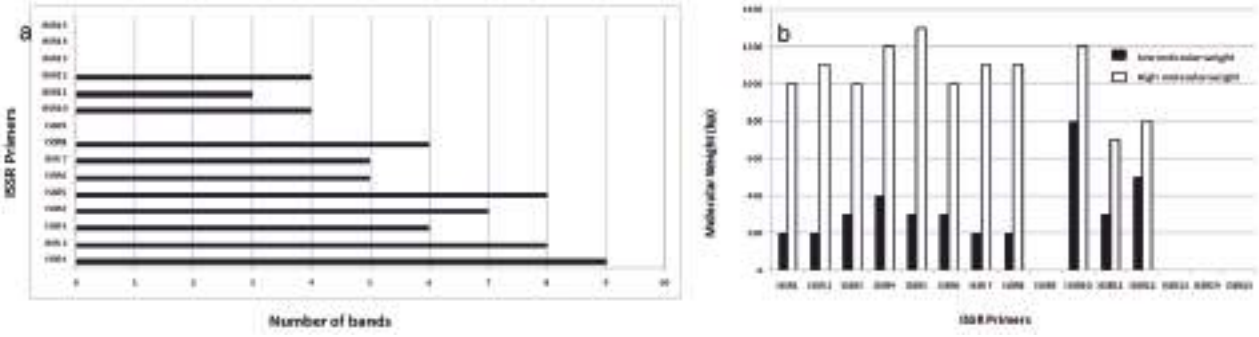


Figure 3. Screening of ISSR primers: fifteen ISSR primers showing the number of amplicons (a) and low and high molecular weight amplicons from the pooled genomic DNA of *coffea congensis* and *coffea canephora*



found to be polymorphic (either present or absent in less than 95% of selected genotypes). We found that one loci of RAPD and three loci of ISSR were monomorphic with an average of 5.33 and 7 per RAPD and ISSR primer. RAPD data revealed that the frequency of polymorphism varied with primers. One of the primers (OPAB3) had produced 100% polymorphic loci. A similar result was observed in the experiment involving ISSR primers that exhibited a frequency of 80 - 85% polymorphic loci (Table 5).

Performance of ISSR markers

The information on genetic profile of *C. congensis*, *C. canephora* and their hybrid derivatives obtained using five selective ISSR primers were used to assess the marker performance through evaluation of four parameters: PIC, EMR, MI and RP (Table 6). To determine the PIC values for all loci of each primer, the mean of PIC values for all the loci of each primer was considered. The range of PIC for three polymorphic loci of RAPD markers was 0.25 – 0.55 and for ISSR markers 0.28 to 0.40 with a mean value of 0.28 and 0.33 for RAPD and ISSR markers, respectively. Three of the polymorphic loci were highly informative (PIC>0.45) while using RAPD primers. Also, 20 ISSR loci had produced higher frequency of polymorphism and these loci were highly informative (PIC >0.45). When the average polymorphic loci were correlated with PIC value data for individual loci, it was found that fragments falling within the 3.5 -7.0 were highly informative.

Effective multiplex ratio (EMR) depends on the number of polymorphic loci. In this study, the highest effective multiplex ratio was observed with the RAPD primer OBAP-03 (EMR 7) and ISSR primers ISSR 1 and ISSR 2 (7.32 and 7.31, respectively) with mean value of 4.77 and 6.12 respectively. To determine the

Table 5. Comparative marker parameters for RAPD and ISSRs for identification of C x R coffee hybrid

Marker Parameters	Values	
	RAPD	ISSR
Total number of primers screened	25	15
Primers producing more polymorphism	03	05
Total number of loci scored	10	26
Total number of polymorphic loci	09	23
Size of amplified bands range (bp)	250-2800	200-1300
Average number of polymorphic loci per primer	5.33	7.22
Percentage of total loci which are polymorphic	90	88

Table 6. Evaluation of RAPD and ISSR markers based on parameters (PIC, EMR, MI and RP)

RAPD/ ISSR primers	PIC		EMR		MI		RP	
	RAPD	ISSR	RAPD	ISSR	RAPD	ISSR	RAPD	ISSR
OPAB – 03	0.36	--	7	--	2.52	--	5.2	--
OPAG – 12	0.25	--	1.33	--	0.532	--	1.50	--
OPAL – 15	0.55	--	6	--	3.30	--	4.75	--
ISSR1	--	0.34	--	7.32	--	2.48	--	5.61
ISSR2	--	0.28	--	7.31	--	2.04	--	4.04
ISSR6	--	0.40	--	4.23	--	1.66	--	3.62
ISSR9	--	0.32	--	5.41	--	1.72	--	4.41
ISSR13	--	0.32	--	6.43	--	2.04	--	4.82
Minimum	0.25	0.28	1.4	4.23	0.532	1.66	1.50	3.63
Maximum	0.55	0.40	7	7.32	3.30	2.48	5.2	5.62
Mean	**0.386**	**0.33**	**4.77**	**6.12**	**2.11**	**1.99**	**3.81**	**4.48**

usefulness of markers, we calculated the marker index (MI) for each RAPD and ISSR primer. The highest MI was observed in OPAG-15 (3.30) and lowest in OPAL-12. In case of ISSR markers, highest MI value was obtained with ISSR 1 (2.48) and lowest with ISSR 6 (1.66).

The resolving power (RP) is a parameter that indicates the discriminatory potential of the primer chosen. The average RP was 2.81 for RAPD and 4.48 for ISSR markers and the highest RP was recorded with RAPD primer OPAB3 (5.2) and lowest with OPAL12 (1.50). It was observed that ISSR 1 has shown the highest RP (5.61) and ISSR 6 thelowest RP (3.62). In addition, some of the primers have been validated for their efficiency to develop into a ISSR based DNA marker in C x R coffee hybrid.

Inheritance of unique RAPD and ISSR marker

The present study revealed that unique RAPD and ISSR markers found in either of the parents could inherit into F1 hybrid. Based on the results, OPAB-03 and OPAG-12 had amplified unique DNA bands with the molecular weight of 1100 bp and 1300 bp in *C. congensis* (female parent) and *C. canephora* (male parent), respectively. These markers were constantly inherited to C x R hybrid and its progenies. OPAG- 15 had amplified one unique band in *C. canephora* (male parent) with 500 bp, found to inherit into F1 and backcross progenies (Fig. IV). ISSR markers, namely, ISSR 1, ISSR2, ISSR6 had amplified unique bands with a size of 900 bp, 1000 bp, 500bp in female parent (*C. congensis*) and these were constantly inherited into F1 and backcross progenies. Similarly, ISSR9 which amplified unique DNA band with 600 bp in male parent (*C. canephora*) constantly inherited to F1 and its backcross progeny (Fig. V). ISSR13 had amplified a faint DNA band with 700bp in F1 and backcross progeny but not found in either of the parent. All the unique bands scored in this study were constantly inherited to F1 and its backcross progenies to use them as useful DNA markers.

Figure 4. Genetic fingerprints of female parent (*coffea congensis*) and male parent (*coffea canephora*), F1 hybrid and backcross progenies of C x R hybrids. Genetic finger prints were developed using three primers OPAB3 (a), OPAG 12 (b) and OPAL15 ©.

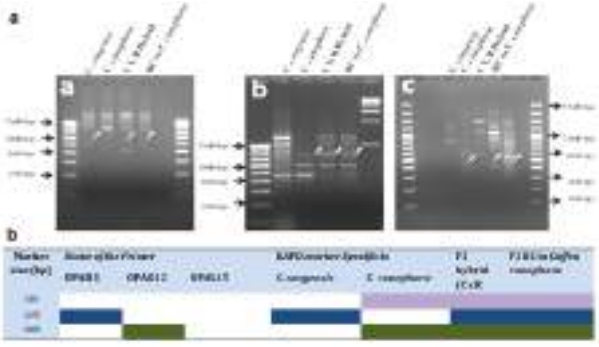
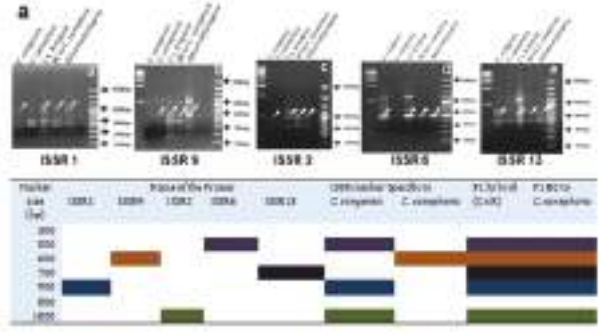


Figure 5. Genetic fingerprints of female parent (*coffea congensis*) and male parent (*coffea canephora*), F1 hybrid and backcross progenies of C x R hybrids. The finger prints were developed using five ISSR primers ISSR1 (a), ISSR9 (b), ISSR2 (c), ISSR6 (d) and ISSR13 (e).



Discussion

*C. congensis*is a smaller bush and known for superior coffee flavour among the diploid species of *Coffea* (Prakash and Ahmad, 1998). But *C. canephora*, another diploid origin is known for higher productivity but possesses inferior aroma quality (Mishra *et al.*, 2011; Koshiro *et al.,* 2007). Genetic

improvement in diploid species of *Coffea* for enhancement of aroma quality is one of the breeding objectives in India and other coffee growing countries (Anagbogu *et al.,* 2019). Crosses were made between *C. congensis* and *C. canephora* since 1942 and their progenies were evaluated for more than 17 years to release a hybrid, popularly known as C x R hybrid (Prakash and Ahmad, 1998). The F1 progeny was intermediate in plant stature with improved aroma than *C. canephora* but with lesser productivity (Prakash and Ahmad, 1998). To improve the productivity, backcross was performed with *C. canephora*. This hybrid is clearly distinguishable from either of the parents during the early stage of growth under field condition. However, after 15 – 20 years of cultivation, the vegetative features often resemble to *C. canephora*.

Mixed cultivation of C x R hybrid and *C. canephora* (one of the parents of C x R hybrid) was recommended in commercial plantation in India as the latter has been realized as an effective pollen donor to C x R hybrid for higher fruit set and productivity (Jamsheed *et al.*, 1996). However, due to varying behavior of vegetative growth of C x R hybrid under different environmental conditions, distinction of C × R hybrid from *C. canephora* became one of the constraints during the plant selection process for seed production and cloning. To support this, different bush sizes of C x R hybrid, namely *C. congensis* type (compact bush with smaller leaves) intermediate type (bush size is intermediate to *C. congensis* and *C.canephora*) and *C. canephora*type (bushes are similar to *C. canephora* with broader leaves) were documented. (Jamsheed *et al.*, 1996; Jamsheed *et al.*, 1998) Unauthentic sources of clones of C x R hybrid with a close resemblance to *C. canephora* often pose a serious problem in precise identification of C x R hybrid during a routine selection process for planting and breeding exercise.

Genetic compatibility among different species of *Coffea* for development of inter-specific hybrids has been well established by surpassing the ploidy barriers between diploid and tetraploid species of *Coffea* (Louarn 15th International Science Colloquium on coffee. ASIC, Paris Charrier; Memories Orstom, Paris 1978; Gomez *et al.*, 2016). For example,Hibrido de Timor popularly known as HdeT (a natural hybrid of *C.arabica*and *C. canephora*) and S-26 (a natural hybrid of *C. liberica*×*C. arabica*) (Anonymous, 1988).HdeT (Hibrido de Timor) and its derivatives became the core gene pool for breeding several arabica varieties of coffee for leaf rust resistance (*Hemileia vastatrix*) as a only source of genes in arabica coffee breeding program (Charrier Eskes Les cafeiers; CIRAD et Orstom, Paris, 1997; Van der Vossen *et al.*, 2015; Alves *et al.,*2024)**.**

In India, S.26 was the first inter-specific hybrid which offers durable resistance to coffee leaf rust disease owing to genetic introgression between *C. liberica,* a diploid origin offering resistance to coffee leaf rust disease and *C. Arabica,* tetraploid origin predominantly susceptible to coffee leaf rust disease (Prakash *et al.,* 2002). However, genetic improvement of diploid species of *Coffea* has not been paid much attention as diploid species of *Coffea* are highly tolerant to major pests and diseases. (Van Der Vossen and H.A.M. London, 1985; Filho *et al.,* 1999; Luis Fernando and Matthew, 2022). Nevertheless, breeding among diploid species of *Coffea* is one of the priorities in many robusta coffee growing countries to improve the aroma quality. (Leroy *et al.,* 2006; Kumar *et al.,*2020). A number of DNA markers have been developed to characterize wide range of coffee genetic resources using RAPD (Agwanda *et al.,*1997; Aga *et al.,* 2003; Maluf *et al.,* 2005; Ramadiana *et al.,*2021; Wahibah *et al.,* 2023; Silvestrini *et al.,* 2008; Kathurima *et al.,* 2011; Achar *et al.,* 2015) and ISSR (Paulo *et al.,*2003; Lin *et al.,* 2019; Panaligan *et al.*, 2020; Mishra

et al., 2011; Tshilenge *et al.*, 2009)markers to understand the genetic relations among the diploid and tetraploid species of *Coffea,* including the inter-specific origins.In addition, RAPD and ISSR markers are applied in breeding programs such as identification of inter-specific hybrid (*C. canephora* x *Coffea arabica*), known as 'Arobasta hybrid' (Ruas *et al.,*2003; Gimase *et al.,* 2019). In India, hybrid specific SNPs as DNA marker was developed for an inter-specific hybrid (*Coffea congensis* x *Coffea canephora*) using two DNA barcoding locus, namely *rbc*L and *mat*K (Nandhini *et al.,*2013).

A detailed studies on genetic diversity of various species of *Coffea* using RAPD and ISSR markers revealed that *Coffea congensis* and *C. canephora* are closer to each other than other species of *Coffea* (Mishra, Nishani and Jayarama, 2011; Silvestrini*et al.,* 2008). This suggests that *C. congensis* and *C. canephora* are freely inter crossable in nature to develop fertile hybrids. Therefore, possibilities of genetic dilution in addition to new recombination among the diploid species of *Coffea* cannot be ruled out. Most importantly, there are substantial evidences that phenotypic characters change under different environmental conditions (Nguyen *et al.,* 2015) besides changing the coffee quality. (Leroy *et al.,* 2006; André *et al.,*2018). This is true in a perennial crop like coffee as the plantation is exposed to wide range of micro and macro environmental condition. For example, C x R hybrid of coffee under varying agro-climatic regions in India often experiences the phenotypic variations (Raghu *et al.,*2003). In the above context, parental and hybrid specific ISSR markers are expected to be highly useful as genetic markers for precise identification of C x R hybrid in a number of applications relating to the genetic improvement.

India is one among the coffee growing countries, thriving to improve the bean quality of *C. canephora* involving the most compatible and better source of genes for coffee quality from diploid origin of *Coffea*. This effort had led to development of a popular C x R variety with acceptable quality of coffee bean than *C. canephora* (https://www.indiacoffee.org). A similar effort involving a cross between *C. arabica* x *C. canephora* led to the improvement of aroma quality of coffee (Priolli*et al.,* 2008). Our study demonstrated parental specific ISSR markers linked to both the parents of C x R hybrid (*C. congensis* and *C. canephora* as female and male parent respectively) and their inheritance into advanced progenies.By repeated PCR amplification using selective ISSR primers, these markers are detectable with high degree of reproducibility.

Previously, we have identified specific SNPs in chloroplast genes of *C. congensis* and *C. canephora* in *rbc*L and *mat*K locus and found that these SNPs are detectable in F1 and backcross progenies of C x R hybrid and its progenies (Nandhini *et al.,* 2013). However, utilization of these SNPs as marker is time consuming as it requires DNA sequencing analysis. Our present study has led to development of alternative DNA markers for precise identification of C x R hybrid to utilize in genetic improvement program. We also confirmed that these markers are tightly inherited in advanced progenies of C x R hybrid for utilizing them as a potential DNA marker. Micro satellites and SNPs can be utilized for high-throughput genotyping of large number of individual plants for a number of applications in the context of genetic improvement. However, the use of these markers is limited due to higher costs, which remains a concern. More recently, a number of developments have been reported on genetic improvement of diploid origin of *Coffea* from developing countries for sustainable cultivation (Davis *et al.,* 2023) including for bean and aroma quality (Bertrand *et al.,* 2023; Bahtiar *et al.,* 2023; Cheserek *et al.,* 2020) and disease resistance and productivity (Akpertey *et al.,* 2022; Alkimim *et al.,* 2021). Therefore, the relatively

cheaper DNA markers like ISSR are highly useful in marker assisted selection.

Conclusion

C x R hybrid and other diploid varieties of coffee (*Coffea canephora*) are conventionally propagated through seeds and cloning of orthotropic shoots. Therefore, ISSR markers, described in this study, are useful to identify the progenies to ensure seed purification/certification in addition to determine the genetic purity during parental selection in breeding process.

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