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Genetic Diversity Appraisal in Robusta Coffee (*Coffea canephora* var. S.274) Beans using Random Amplified Polymorphic DNA Markers

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ABSTRACT: Coffee is a most extensively accepted beverage for many centuries. As an export-oriented commodity, coffee plays key position in the national economies of nearly 80 developing countries in the world involved in its production. Indian Coffees are renowned as the world's best shade-grown 'mild' coffees. Several studies conducted earlier have signified that molecular biology approaches based on DNA detection methods play a dynamic role in varietal identification, food safety control, and authentication. Molecular markers have been widely useful over recent years to endow with a better perceptive of the genetic basis of important agronomic traits in plants. Breeding programs in coffee are more concerned with the identification of agronomic traits such as quality, resistance to major pests, and diseases besides productivity. In coffee biotechnology, Random amplified polymorphic DNA (RAPDs) are useful in cultivar identification, population & pedigree analyses, phylogenetic studies and genetic mapping. In the present investigation twenty five decamer primers were used of which twelve primers gave consistent and clear amplification products. These twelve primers gave consistent and clear amplification products, of which 70.4 percent bands were polymorphic and the remaining 24 percent bands were found to be monomorphic. On average each primer amplified average of 6.75 bands, of which 4.75 bands were polymorphic. The RAPD amplification has revealed that, Bot 18, Bot 22, and Bot 24 amplified four rare alleles in the samples analyzed. Based on the RAPD pattern it revealed that, relatively low genetic diversity in Robusta coffee (*Coffea canephora* var. S.274) gene pool. These results also indicate the specificity of RAPD characterization of *Coffea canephora* (Robusta coffee), and this detection system assay can be utilized as a promising molecular tool for future breeding programs; exploring innovative prospects in food authentication and traceability.

Keywords: Robusta Coffee, *Coffea canephora*, RAPD, Genetic Diversity, Organic and Integrated nutrition management (INM) practices.

INTRODUCTION

Coffee is the most widely consumed and most popular drink in the world. It is also an extensively traded tropical product for many centuries and is renowned for its unique flavor and health benefits (Dankowska *et al.*, 2017). It is one of the most important articles of international trade and raw material traded throughout the world subsequent to crude oil. It has become the most important export article for the nations that grow it. Indian coffee is the most astonishing of beverages, contributing intriguing subtlety and stimulating intensity. Further, India is the only country that grows all of its coffee under natural shade and is renowned as the world's best shade-grown 'mild' coffee. These coffees are typically mild and not too acidic; possess an exotic full-bodied taste and a fine aroma. Coffee is an important agricultural product in the world market and is widely grown as a tropical tree crop in about 50 countries. India accounts for about 4.5 percent of world coffee production and the industry provides employment to a 6 lakh workforce. During FY 2020 – 2021, India has produced 342,000 MT of Coffee and Exported 16410 MT to 50 different countries, and gained foreign exchange of Rs. 278905 lakhs during FY 2020 – 2021 exchequer, which include Italy (33435 MT - 20.37%), Germany (33435 MT - 11.72%), Belgium (13901 MT - 8.47%), Russian Federation (7823 MT - 4.77%) and U.S.A. (3813 MT - 2.32%) etc. (Coffee Statistics, 2021).

Both Arabica and Robusta coffee varieties are commercially cultivated in India, owing to the advantage of their assorted ecological adaptation to high and low altitudes correspondingly. Robusta coffee is native to West Africa, reported to have been established in India from Java during 1900–1905 AD (Anonymous, 1985). Robusta coffee is the low land coffee that is richly endowed with several economically valuable traits like high tolerance to leaf rust pathogen, white stem borer, nematode invasion along potential to give consistently high yields. Conversely, Robusta coffee is having pessimistic characteristics such as the inability to endure long drought, small bean size, and

lack of good flavour in the beverage quality. Hence, the main objectives of Robusta breeding in India have been directed mainly towards evolving high-yielding, widely adaptable varieties with improved bean and quality traits. The coffee plant is a perennial and evergreen in nature. The coffee plant belongs to the *Rubiaceae* family, which has some 500 genera and over 6000 species (Ukers, 2012). *Coffea* genus, subgenus *Eucoffea* and comprises more than 90 different species, of which 25 have been more extensively studied (Davis *et al*., 2006). However, commercially only two species are cultivated i.e., *Coffea arabica* (*Arabica coffee*) and *Coffea canephora* (Robusta coffee). The *Coffea canephora* is a diploid species and most extensively cultivated around the world. It is self-sterile and cross-pollinated and consequently displays much more variability than *Coffea arabica*. The Robusta coffee is better adapted to warm and humid equatorial climates and is commonly cultivated in low to medium elevations. In India, the consequences of leaf rust and white stem borer (W.S.B.) on *Coffea arabica* primed Indian planters to introduce *Coffea canephora* (Robusta coffee) during 1903 to 1906. For achieving sustainable eco-friendly coffee production through rust tolerance, high productivity, wide adaptability, and improved quality, Central Coffee Research Institute (CCRI) developed superior and improved Robusta cultivar (S.274) and distributed this variety for commercial cultivation due to characteristic bold beans, wide adaptability alongwith high yield potential (Anonymous, 2014).

Breeding programs in coffee are more concerned with the identification of agronomic traits such as quality, resistance to major pests, and diseases besides productivity. Efforts are made to achieve these objectives amidst several limitations especially in selecting the suitable mother plants to be used in the breeding exercise. In this vision, the approach of molecular markers is one of the powerful tools that can be used for identifying and differentiating various genetic stocks. The impact of PCR on molecular biology has been profound since the reaction is easily complete and leads to the amplification of specific DNA sequences by a massive factor. From a simple basic principle, many variations have been developed with applications throughout gene technology (Erlich and Arnheim, 1992). Molecular markers have been widely useful over most recent years to endow with a better perceptive of the genetic basis of important agronomic traits in plants (Masojc, 2002). The molecular techniques based on polymorphisms in DNA have played a key role in the assessment of genetic variability, catalyzing research in an assortment of disciplines such as phylogenetics, taxonomy, ecology, genetics, and breeding during the last three decades (Weising *et al*., 2005). Molecular markers techniques greatly facilitated the construction of linkage maps, Paillard *et al*., (1996) constructed a molecular linkage map in coffee (*Coffea canephora P.*) by PCR-based markers including random amplified polymorphic DNA (RAPD). Besides, RAPD markers were also used for the identification of the genetic variability within and amid coffee populations (Silveira *et al*., 2003). Many authors employed RAPD markers to identify coffee accessions, the effectiveness of RAPD markers to identify coffee accessions is demonstrated by Lashermes *et al*., (1993) and Orozco-Castillo *et al*., (1994). Further, Lashermes *et al*., (1993) showed that intraspecific variation can be easily detected by RAPD markers, in *C. canephora* and *C. liberica*. Similarly, Lashermes *et al*., (1996) successfully employed RAPD markers to analyze the genetic diversity among cultivated and sub-spontaneous accessions of *C. arabica* and confirmed the narrow genetic base of commercial cultivars. Lashermes *et al*., (1996a) also studied the Inheritance and genetic mapping of self–incompatibility in *C. canephora Pierre* and concluded that the availability of a linked DNA marker should facilitate the genetic analysis of self-incompatibility in relation to coffee breeding programs. Further, molecular tools utilizing DNA markers have to be exploited to increase perceptive of coffee genetic diversity and to develop strategies for the conservation of coffee genetic resources with wide genetic representation (Krishnan, 2011).

Recently, DNA analysis through molecular markers has unlocked an innovative way to cognize complex organism's genomes. DNA analysis is widely applied across different fields as an ideal biological tool due to its unique characteristics such as its presence in most of the biological tissues, it can be amplified initially from very low quantities and it is more stable than other molecules such as proteins towards physical and chemical processes in processed food. Therefore, DNA is serving as a fascinating target for traceability purposes in food control (Martins-lopes *et al*., 2013). Several studies have indicated that Random amplified polymorphic DNA (RAPDs) are useful in cultivar classification, population and pedigree analyses, phylogenetic studies, and construction of genetic maps (Halward *et al*., 1992). Congruently, Combes *et al*., (2018) developed a rapid and efficient DNA-based authentication method to detect and quantify adulterations in coffee (Arabica versus Robusta) which overcome limitations of metabolic profiling of Robusta and Arabica coffees. The RAPD technique has been used in coffee to study the genetic diversity and relationships among the *Coffea* species these studies disclosed narrow genetic base characteristics of the cultivated varieties within *Coffea arabica* species (Lashermes *et al*., 1996 b; Orozco-Castillo *et al*., 1994). Conversely, studies conducted by Lashermes *et al*., (1993); Orozco-Castillo *et al*., (1994) also confirmed some degree of polymorphism in Rume Sudan and Catimor varieties and interspecific gene introgression between Arabica coffee (*C. arabica*) and Robusta coffee (*C. canephora*). The accessibility of T-linked RAPD markers represents a starting point in the use of markers to enhance backcross programs in Arabica coffee. After further investigation and characterization on a large F2 population, the identified RAPD markers could be converted into co-dominant PCR-based

sequence-tagged site (STS) markers for utilization in marker-assisted selection (Paran & Michelmore, 1993). Potential targeted genes comprise imperative traits such as resistance to root-knot nematodes, leaf rust, and bacterial blight of coffee (Moreno, 1989). Further, specific genotypes of coffee manifest resistance to specific sets of rust races (Ram and Sreenath, 2000). However, currently internationally validated method to confirm or otherwise the correctness of a claim of a specific country of origin for a particular sample of coffee is not available. The use of molecular markers is advised, since studying the usual trace element pattern for a given region may be disturbed using fertilizers. However, due to postharvest treatments, the reliability of molecular markers for identifying specific countries rests on the degree of forensic robustness of the authenticity of location for the samples used to produce particular data sets toward a comparison with samples with variations (Burns and Walker, 2020). Therefore, molecular biology approaches based on DNA detection methods play a dynamic role in the field of food safety control and authentication. These DNA-based methods allow the identification of species as well as their relative quantification. They are generally ideal for food products and are considered highly reliable since DNA is very stable compared to other molecules under high temperatures, pressures, and chemical treatments used during food processing (Madesis *et al*., 2014). However, a suitable protocol is required for DNA extraction, isolation of DNA free of inhibitors, sufficient quantity, and quality. Therefore, the DNA extraction method necessities optimization, because DNA status can influence the PCR amplification (Martins-Lopes *et al*., 2013). However, most of the studies conducted earlier to evaluate organic food quality are based on the measurement of selected food compounds (parameters) comparing food from organic versus non-organic origin and differentiation on the genetic pattern is lacking. Further, species detection in coffee products is imperative to protect consumers from adulteration. Considering the ample geographical distribution of Robusta coffee, characterization and evaluation of Robusta coffee gene pool is necessary for effective crop improvement programs and also for better conservation and management of genetic resources. This study aimed to characterize the RAPD profile of Robusta coffee (*C. canephora* - S.274) beans by using the randomly amplified polymorphic DNA profiles (RAPD-PCR) technique to assess the genetic diversity.

MATERIALS AND METHODS

Study Location: This field experiment was carried out at nine selected Robusta coffee estates located at Western Ghats of India, i.e., Koppa region of Chikkamagaluru District, Chikkamagaluru district is situated in the southwestern part of Karnataka State, between 12º54′ and 13º53′ north latitude and between 75º 04′ and 76º 21′ east longitudes. 2,509 m above sea level, with an average mean annual Rainfall of 2908 mm. The tropical climate prevails in the study location, the relative humidity ranges from 27 to 80% and the average wind speed ranges from between 4 to 7 km/hr. The climate in the study location having three distinct seasons; 1) Summer season - March to early June, 2) Monsoon season – early June to September, however very little rainfall occurs during October to November due to impact North East Monsoon, 3) winter season generally initiates in mid - November and ending in mid - February. Among the selected 9 coffee estates, four estates practice organic mode of nutrition, while four estates pursued integrated nutrition management (INM) practice and one estate where no nutrition management is practiced (complete control). Varying shade patterns (open and thick) and irrigation (blossom, backing, and winter) are the delineation factors in the selected coffee estates practicing exclusive organic cultivation and integrated nutrient management. The present field experiment was laid out in randomized block design (RBD) with 25 plants per treatment (plot size- 112 m^2) with four replications. The selected estates under organic cultivation were practicing organic farming for the preceding four years. The other cultural practices were carried out as per the standard package of practices as recommended by the Coffee Board of India (Anonymous, 2014). The treatment details are as follows

T1- Control

T2 - Organic nutrition*, thick shade (TS - 50 to 60% canopy) + Irrigation - I (winter)

T3 - Organic nutrition*, thick shade (TS - 50 to 60% canopy) + Irrigation -II (Blossom & Backing)

T4 - Organic nutrition*, optimum shade (OS - 25 to 30% canopy) + Irrigation - II (Blossom & Backing)

T5 - Organic nutrition*, optimum shade (OS - 25 to 30% canopy) + Irrigation - I (winter)

T6 – INM#, thick shade (TS - 50 to 60% canopy) + Irrigation - I (winter)

T7 - INM#, thick shade (TS - 50 to 60% canopy) + Irrigation (Blossom & Backing) - II

T8 - INM#, optimum shade (OS - 25 to 30% canopy) + Irrigation – II (Blossom & Backing)

T9 - INM#, optimum shade (OS - 25 to 30% canopy) + Irrigation - I (winter)

* **Organic nutrition** -100% organics [Farm Yard Manure and Compost -2.5 tones ha^{-1}, Rock phosphate 0.2 tones ha^{-1}],

Integrated nutrition [50% recommended dose of fertilizer (Anonymous, 2003) + 50% organic manures]

Winter-irrigation (I): At least four irrigations at winter, blossoming, backing and summer (interval of twenty days), extended if dry spell continuous

Blossom backing irrigation (II): Irrigations at blossoming and backing

Experimental Design and sample collection: The present experiment was laid out in randomized block design (RBD) with 25 plants per treatment (plot size- 112 m^2) with four replications. The organics estates were selected where organic farming practices were practiced in the preceding four years. Representative coffee fruits from all nine Robusta coffee growing estates were collected during the harvesting period

(February to March). After harvesting the fruits they were wet-processed to remove pulp and mucilage from the fruits. Further, they were sun-dried up to 10 percent moisture level and stored using standard methods followed in parchment coffee (Anonymous, 2014).

Coffee Bean samples collection and processing: Representative coffee fruits from all nine Robusta growing coffee estates were collected during the harvesting period (February to March). After harvesting the fruits, they were wet-processed to remove pulp and mucilage from the fruits. Further, they were sun-dried up to 10 percent moisture level and stored using standard methods followed in parchment coffee (Anonymous, 2014).

DNA Extraction from Green Coffee Bean and PCR Amplification:

(a) Sample Preparation: About fifty grams of dried seeds of different coffee samples were crushed in a mill and powdered by using a domestic grinder. The powder was sieved using thin mesh and finely ground powder was kept in the refrigerator until DNA extraction. The seed DNA was extracted as a method followed by Mishra *et al*., (2008).

(b) Bean RAPD analysis: After DNA extraction from the seeds, the Polymerase Chain Reaction (PCR) amplification was done using random primers (RAPD). The amplified products of PCR were electrophoresed on an agarose gel (1.5%) pre-stained with Ethidium Bromide (0.5 µg/ml) in 1X TAE (Tris Acetic EDTA) buffer and visualized using Syngene Gene snap as described by Mishra *et al*., (2008).

Sample details

Lane1- Organic old sample; Lane 5- Control (T1) ; Lane 9- Organic (T5)

Lane 2- Organic old sample; Lane 6– Organic (T2) ; Lane 10- INM (T6)

Lane 3- INM old sample; Lane 7- Organic (T3) ; Lane 11- INM (T7)

Lane 4- INM old sample; Lane 8- Organic (T4) ; Lane 12- INM (T8) ; Lane 13- INM (T9)

RESULTS AND DISCUSSION

Assessment of genome diversity in a gene pool of any particular species offers great promise for better exploitation and management of genetic resources. Green coffee beans cultivated by organic and integrated nutrition were selected in the present investigation since green coffee beans do not undergo extensive processing and this can preserve the DNA molecule intact, which positively influences the real-time PCR results (Fernandes *et al*., 2013). The coffee bean samples from 13 different Robusta coffee (organic and INM) growing estates were subjected to Random Amplified polymorphic DNA (RAPD) analysis to understand the genetic relatedness and differences among samples that are subjected to different treatments. Twenty-five decamer primers were used of which twelve primers gave consistent and clear amplification products. These 12 RAPD primers amplified 81 bands in 13 samples of which 57 bands (70.4 percent) were polymorphic and the remaining (24) were monomorphic. Each primer amplified the different number of fragments varied from 2 to 13 with an average of 6.75 bands amplified by a primer. The polymorphic bands amplified by a primer varied from 1 to 10 with an average of 4.75. The RAPD amplification has revealed 4 rare alleles in the samples analyzed. The rare alleles were scored in Lane-11 (INM-T7), Lane-1 (organically grown Robusta old samples), and Lane-5 (Control). The primers namely, Bot 18, Bot 22, and Bot 24 amplified these rare alleles. The frequency occurrence of various bands varied and the same is given in Table 1.

Table 1: Primers and their sequences used in the RAPD- PCR study.

Sr. No.	Primer	Primer Sequence 5^1- 3^1	Total No. of bands	No. of polymorphic bands
1.	Bot 09	GGGAGACATC	6	4
2.	Bot 11	GAACGGACTC	7	5
3.	Bot 12	TGGACCGGTG	7	6
4.	Bot 13	AAAGCTGCGG	10	8
5.	Bot 17	ACCGCGAAGG	5	4
6.	Bot 18	GGGGTGACGA	7	6
7.	Bot 20	GAACACTGGG	7	4
8.	Bot 21	GGGAATTCGG	6	3
9.	Bot 22	GGGCGGTACT	6	3
10.	Bot 24	ACCACCCACC	13	10
11.	Bot 41	TGGCGCAGTG	5	2
12.	Bot 43	TGGCAAGGCA	2	1
Grand Total			**81**	**56**

Based on the RAPD banding pattern, genetic affinities between the samples were given in Table 2. The samples which are under organic and INM treatments (samples 6 to 13) showed high affinity among them, the control (sample 5) showed comparatively lower affinity towards other treatments. The old Robusta samples from organic and INM samples showed a lesser genetic affinity with other treatments. In contrast, the old Robusta samples (samples 1 to 4) exhibited high affinity among themselves.

Polymorphic bands and rare alleles may represent the uniqueness of the varieties and could be due to many factors including the change in genetic sequence in coding or non-coding regions or SNP (Single nucleotide polymorphism) or recombination events during meiosis and crosspollination. Similarly, previous studies had also concluded that RAPD analysis is a reliable and effective method to evaluate genetic variability within and amid *Coffea* species (Orozco-Castillo *et al*., 1994; Maluf *et al*., 2005). The RAPD markers linked to genes of interest are also utilized for coffee genetic improvement such as identification of RAPD markers for resistance to coffee berry disease (Agwanda *et al*., 1997) and for Coffee Leaf Rust Differentials (Ram and Sreenath, 2000). Since the knowledge of genetic dissimilarities and the genetic affiliation amid genotypes is an important deliberation for proficient rationalization and exploitation of germplasm resources (Russel *et al*., 1997). In this perspective, the findings of the present study have high significance for efficient exploitation of variability in the breeding programs and the easy management of genetic resources.

Table 2: Correlation of genetic relation of Robusta coffee.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1	X	0.76	0.76	0.79	0.66	0.58	0.55	0.52	0.49	0.44	0.51	0.49	0.47
2		X	0.81	0.72	0.80	0.61	0.63	0.55	0.63	0.59	0.57	0.62	0.59
3			X	0.69	0.69	0.71	0.73	0.60	0.61	0.61	0.53	0.63	0.57
4				X	0.72	0.64	0.60	0.53	0.53	0.56	0.45	0.62	0.52
5					X	0.67	0.76	0.76	0.68	0.72	0.59	0.62	0.62
6						X	0.83	0.68	0.65	0.69	0.72	0.65	0.81
7							X	0.67	0.80	0.82	0.77	0.78	0.74
8								X	0.79	0.78	0.72	0.70	0.76
9									X	0.88	0.77	0.86	0.83
10										X	0.86	0.80	0.88
11											X	0.79	0.87
12												X	0.98
13													X

CONCLUSION

In the present study, DNA extraction was done from the green coffee of Robusta coffee (*Coffea canephora* var. S.274) beans. Further by DNA samples were subjected to Polymerase Chain Reaction (PCR) and amplification was done using random primers (RAPD) analysis. Twenty-five decamer primers were used to understand the genetic relatedness and differences among samples that are subjected to different treatments. These twelve primers gave consistent and clear amplification products, of which 70.4 percent bands were polymorphic and the remaining 24 percent bands were found to be monomorphic. On average each primer amplified an average of 6.75 bands, of which 4.75 bands were polymorphic. The RAPD amplification has revealed that Bot 18, Bot 22, and Bot 24 amplified four rare alleles in the samples analyzed. Hence only one variety of Robusta coffee (S.274) is chosen for the present study, RAPD pattern is also revealed relatively low genetic diversity in the Robusta coffee (*Coffea canephora* var. S.274) gene pool. Therefore, insignificant differences between banding patterns in the different treatments by PCR studies indicated that the variations observed among the various treatments were exclusively due to distinction between treatments. To study the genome more completely, the future line of work may be focused on investigating green Robusta coffee beans with additional markers elaborately and incorporation advanced molecular biology tools such as amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR) markers, Next Generation Sequencing (NGS - for short DNA targets of 15 to 30 bp) and Loop-mediated Isothermal Amplification (LAMP). The characterization and discrimination of green coffee beans cultivated by different nutrient management may be done by utilizing sophisticated analytical instruments such as Fourier transform infrared (FTIR) spectrophotometry, FT-MIR-PAS, mass-spectroscopy, etc. Thus, the development of personalized methods for food diagnostics will enable accurate quantitative analysis by optimizing the time and economics along with easy-to-perform procedures will unlock exciting opportunities in food authentication and traceability. Further, on the way to the identification of a particular cultivar, amalgamation of botanical, agronomical, and molecular descriptors could be harmonized through the identification of gene polymorphism. The results of the present investigation also indicate the specificity of RAPD characterization of Robusta coffee, and this detection system assay can be utilized as a promising molecular tool for future breeding programs; exploring innovative prospects in food authentication and traceability.

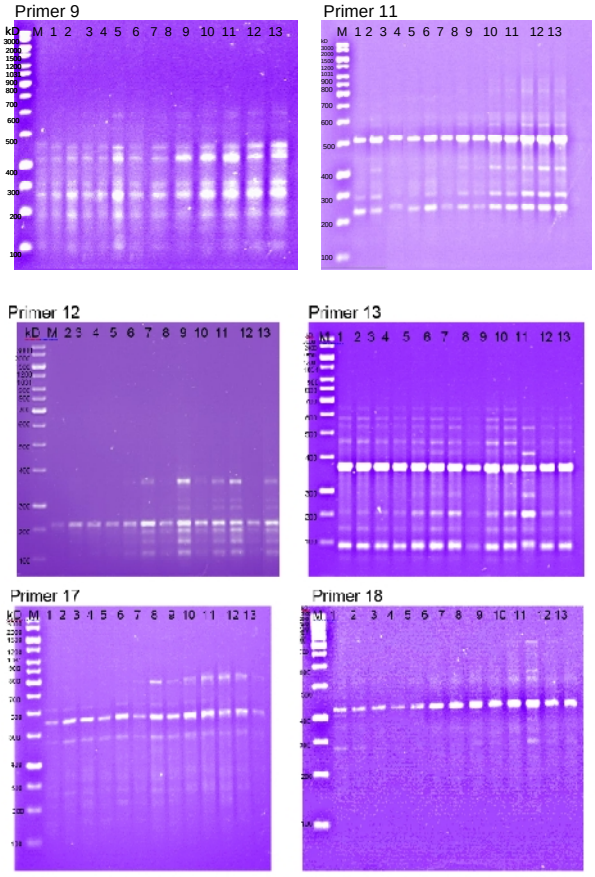

Plate 1: RAPD-DNA banding pattern of coffee beans.

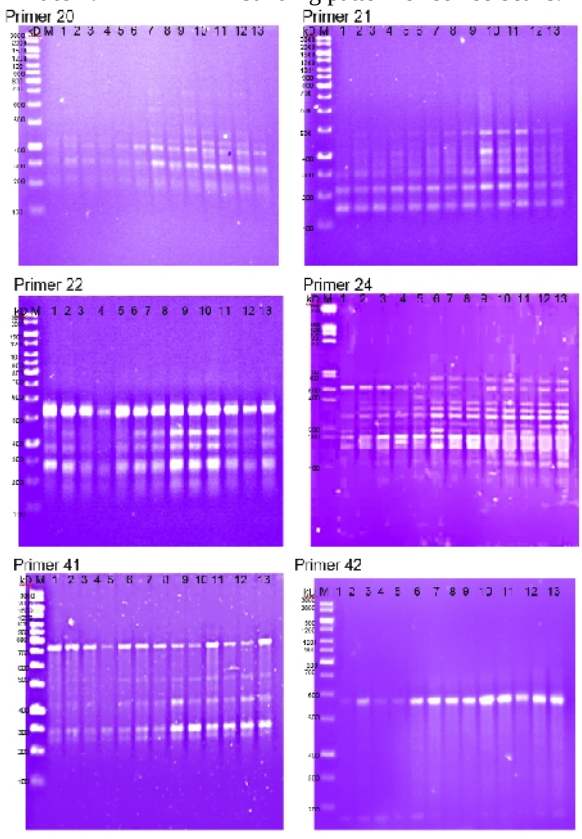

Plate 2: RAPD-DNA banding pattern of coffee beans.

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Conflict of Interest. Nil.

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