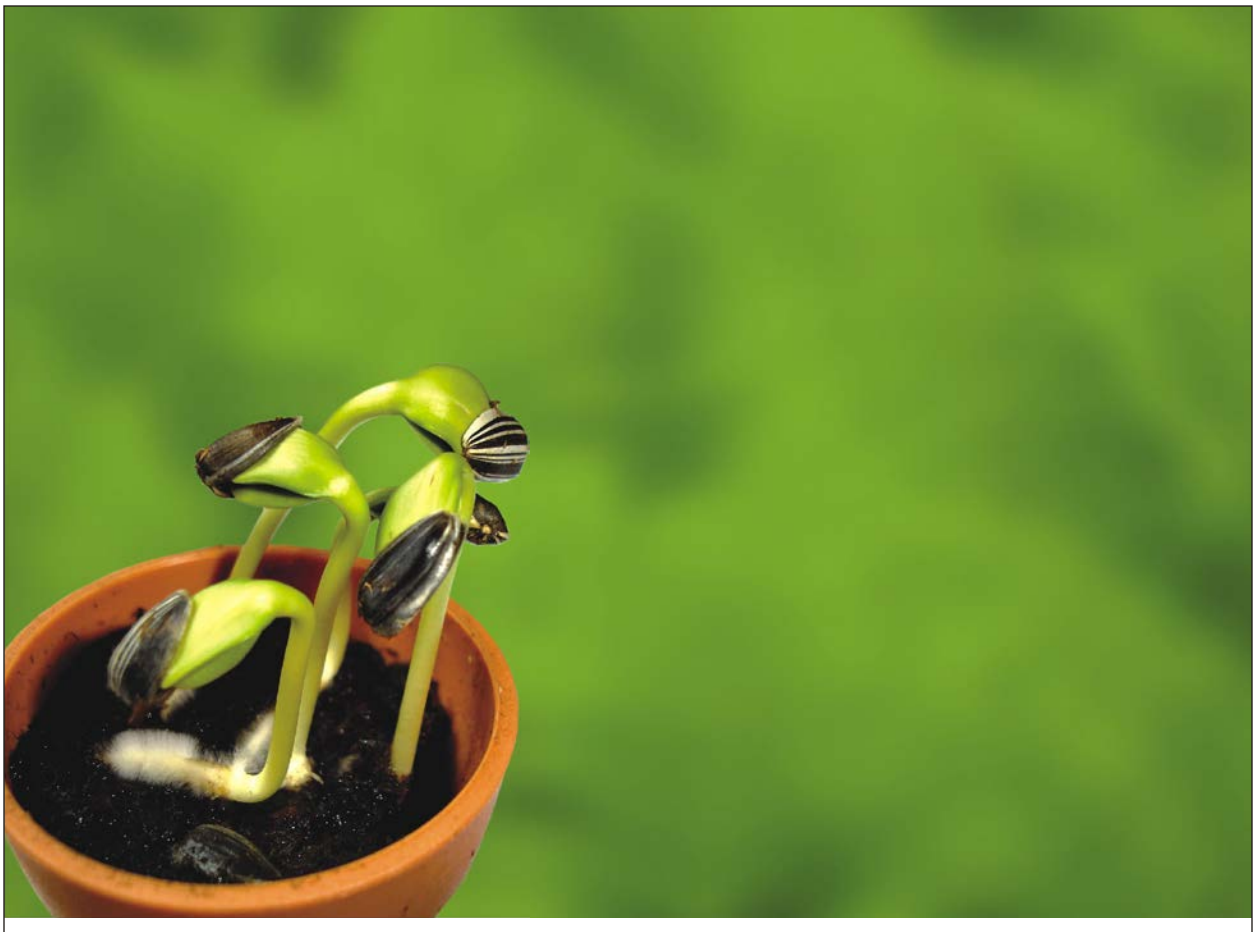

International Journal of Plant Breeding and Genetics

ISSN 1819-3595

Academic Journals Inc.

www.academicjournals.com

International Journal of Plant Breeding and Genetics 9 (3): 95-105, 2015
ISSN 1819-3595 / DOI: 10.3923/ijpbg.2015.95.105
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Study of Genetic Diversity of *Coffea canephora* Pierre ex A. Froehner Accessions by RAPD Markers

[1]A.M. Devaraja Achar, [2]George Danial, [2]Mallikarjuna Awati, [1]M. Udayakumar and [1]T.G. Prasad

[1]University of Agricultural Sciences, GKVK P.O, Bangalore, 560 065, Karnataka, India
[2]Regional Coffee Research Station, Coffee Board, Chundale P.O. Box 673123, Wayanad, Kerala, India

Corresponding Author: A.M. Devaraja Achar, Department of Crop Physiology, University of Agricultural Sciences, GKVK P.O., Bangalore, 560 065, Karnataka, India

ABSTRACT

Random amplified polymorphic DNA (RAPD) markers were used to assess the level of genetic variability among 35 robusta coffee accessions consisting exotic and private estate collections having good yield performance under drought conditions DR (drought resistance) accessions. Seventeen informative RAPD markers were generated 212 loci, of which 122 loci found polymorphic (58%). The dendrogram was drawn to visualize relationships among the accessions, wherein three major clusters were formed, where in exotic collections showed closely relatedness with local estate collections demonstrated the narrow genetic base but no trends of significant genetic variance. Even in the dissimilarity matrix constructed by Squared Euclidean Distances (SED) and three-dimensional Principal Component Analysis (PCA) also conferred close distribution of the accessions. The correlation between dissimilarity matrices generated by these methods (r = 0.3) indicated they are positively associated but the relationship was non-significant. However, Drought Resistance (DR) accessions grouped distinctly, which can be phenotyped and used as genetic material for breeding for drought resistance.

Key words: RAPD, AFLP, ISSR, genetic variability, *Coffea canephora*

INTRODUCTION

Coffee is one of the most important beverages in the world and global consumption has almost doubled in the last 40 years and is forecast to reach 9.09 million ton by 2019. Coffee provides a livelihood for a further 100 million people in coffee-producing countries and Coffee-producing countries earned $23.5 bn from coffee exports in 2011 (fairtrade.net, 2012). The commercial coffee comes from two main species, *Coffea arabica* L. Arabica coffee (2n = 44) and *Coffea canephora* Pierre ex Froehner (Robusta coffee, 2n = 22). Of these two species, arabica coffee accounts for 70% of the market, compared with 30% for Robusta. All *Coffea* species are diploid (2n = 2x = 22) and generally self-incompatible, except for *C. arabica*, which is tetraploid (2n = 4x = 44) and self-compatible. However, the *coffea* species share a common genome, making possible interspecific hybridizations and hybrid production either within or between *Coffea* species (Charier, 1978; Louarn, 1992; Le Pierres, 1995). Similarly, molecular analysis revealed that recombination between chromosomes is not significantly restricted by genetic differentiation between these two species (Herrera *et al*., 2002; Anthony *et al*., 2002). This shows the potential value of genetic resources as sources for transfer of new characters from diploid species into the genome of *C. arabica* cultivars.

Robusta coffee is native to low land forest of the Congo River basin (Africa), shallow rooted, inherently drought susceptible and disease resistance. The disease resistance genes from *C. canephora* have been transferred into *C. arabica* gene pool via natural and inter-specific hybridization between the two species (Leroy *et al*., 2006; Caicedo *et al*., 2013). However, genetic variation within *C. canephora* species is not much known owing to clonal propagation and genepool is conserved in *ex-situ* collection plots in several countries (Tshilenge *et al*., 2009). Conversely, it is known that *C. arabica*, have especially narrow genetic base (Anthony *et al*., 2002) compared to *C. canephora* (Prakash *et al*., 2005; Hendre and Aggarwal, 2014).

Genetic diversity of coffee can be assessed using different techniques that range from the traditional morphological techniques to the modern DNA-based molecular markers. The use of morphological techniques in diversity study of plants is limited by the influence of environmental factors and growth stage of the plant (Bita and Greats, 2013; Gratani, 2014). As DNA based markers are environmental insensitive, relatively easy and low-cost procedure and alternative for conserving specific genes responsible for heritable characteristics of particular value (e.g., disease resistance) (Adams and Adams, 1992; Porth and El-Kassaby, 2014). The major PCR-based marker systems that are currently available for genetic study are RAPID Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeat (ISSR) and Amplified Fragment Length Polymorphism (AFLP), which are generally described as dominant markers. Due to the nature of the loci being found scattered throughout the genome, these dominant markers are generally very useful for assessing genetic diversity and sampling the whole genome randomly (Lowe *et al*., 2004; Weising *et al*., 2005; Idrees and Irshad, 2014). This is because the loci scattered through the entire genome can be rapidly genotyped using PCR techniques (Twyman, 2003; Sharma *et al*., 2014).

The molecular markers such as RAPD, ISSR, AFLP and SSR were deployed in robusta coffee accessions revealed the history of evolution (Orozco-Castillo *et al*., 1994; Poncet *et al*., 2004; Kathurima *et al*., 2012; Ferrao *et al*., 2015), inbred differentiation and accessions outcross (Maluf *et al*., 2005; Motta *et al*., 2014), geographical location (Masumbuko *et al*., 2003) and disease resistance (Diniz *et al*., 2005; Berthaud and Charrier, 1988; Seral *et al*., 2003). The AFLP and SSR markers made known good amount of genetic diversity and inter accession differentiation in Indian robusta coffee genepool. Similarly, SSR markers revealed Ugandan *C. canephora* was found to be highly diverse with a genetic substructure consisting of wild, feral and cultivated populations, implying that they form new groups within the *C. canephora* species genome. Molecular investigations also indicate that *Coffea arabica* is an amphidiploid species containing two genomes that was considered to originate from two related diploid species, C. eugenioides and C. canephora (Lashermes *et al*., 1999). However despite the fact, the wider genetic base was not fully utilized in crop improvement. Existing diversity of cultivars is barely tapped by breeders due to scarcity of information on accessions except geographical origin and taxonomic status and hence need for make use of variability with and among cultivars using genetic diversity methods (Upadhyaya *et al*., 2006). In view of this there is a great urgency for intensifying evaluation and conservation of *C. robusta* gene pool has desirable agronomic traits for crop improvement. Having said that the robusta coffee not well studied at DNA level, our main objective of the present study is to study the variable genetic integrity in exotic and estate collections of robusta coffee accessions with RAPD markers, which can be explorable for breeding programmes.

MATERIALS AND METHODS

Genetic materials: Thirty-five *Coffea canephora* accessions including exotic species were selected at regional coffee research station, Chundale, Kerala, India. The accessions used for the

investigation including ten exotic collections and twenty five from local coffee estates yielded better under drought conditions; JAVA (S. 8791), MADAGASCAR (S. 1932), SAIGAN (S. 1902), UGANDA (S. 880), UGANDA (S. 1979), COSTARICA (S. 3389), GUATEMALA (S. 1481), COSTARICA (S. 3400), IVARYCOST (S. 3855), IVARYCOST, DR_1, DR_2, DR_3, DR_4, DR_5, DR_6, DR_7, DR_8, DR_9, DR_10, DR_11, DR_12, DR_13, DR_14, DR_15, DR_16, DR_17, DR_18, DR_19, DR_20, SIB-4, SIB-7, ROBUSTABOLD, ROBUSTA HIGH YIELD and DWARF CxR. The exotic selections from origins and DR series collection called drought resistant while they have been selected from local kerala local coffee estates performed better under drought condition.

To get true to type of plant material, four months old healthy orthotropic shoots with dark-green foliage were collected during the rainy season (June-July). From the selected shoots, 10 cm length single node cuttings were prepared. To prevent fungal infection, cuttings were treated with a fungicide solution by immersing the cuttings for 15 min. As a pre-planting treatment, IBA solution at 1000 ppm concentration was used as quick-dip application. All the treated cuttings were then inserted vertically into polythene bags containing soil mixture in a proportion of 6: 2:1 (Forest soil: Sand: FYM). These polythene bags were arranged in the propagation trenches and after planting the cuttings; the trench was covered with transparent polythene sheet. These plants were maintained for one year in the nursery trenches for better root establishment. One year after establishment of clones at nursery conditions, the selected single nodal cuttings of 35 accessions were then transplanted to main field.

DNA extraction and RAPD primers selection: The genomic DNA was extracted from dried leaves of 35 *C. Canephora* accessions by following Porebski *et al*. (1997) method modified (Bhat, 2002). The quality test revealed that no DNA samples were degraded. DNA concentrations were ranging 300-400 ng uL^{-1}. Of the 35 RAPD primers, 18 informative primers were selected genotyping the diversity analysis.

PCR amplification: The RAPD primers were purchased from Operon Technologies Alameda, CA, USA. A total of 40 decamer oligonucleotides of arbitrary sequence were tested for PCR amplification. The basic protocol reported by Williams *et al*. (1990) for PCR was performed in a total volume of 15 µL reaction volume containing DNA samples (15 ηg μL^{-1}), 1x buffer, 2 mM dNTPs, 2.5 mM $MgCl_2$, 5 pico moles primer, 1 U *Taq* DNA polymerase (Bangalore Genei, India).

DNA amplification was carried out in a T-100, Biorad and the thermal cycler conditions for PCR reactions were an initial denaturation cycle of 1 min and 30 sec at 94°C was followed by 45 cycles comprising 1 min at 94°C,1 min at 36°C and 2 min at 72°C. An additional cycle of 7 min at 72°C was used for final extension. Amplification products were separated by electrophoresis (8.3 V cm^{-1}) in 1.5% agarose gels and stained in ethidium bromide. A photographic record was taken under UV illumination.

Statistical analysis: The binary data scored for the presence (1) or absence (0) from each RAPD primer in all the 35 accessions were analyzed with the computer package called "STATISTICA". A dendrogram was constructed for both phenotypic and genotypic data using UPGMA (unweighted pair-group method using arithmetic averages) method.

RESULTS

The best 17 primers that rendered polymorphic and reproducible banding pattern were chosen for the DNA polymorphism in 35 Coffee accessions (Table 1). The amplified DNA bands were

Table 1: Primers and their sequences used in the genetic diversity study

Primer	Sequence 5'- 3'	Total No. of bands	No. of polymorphic bands	Polymorphism (%)
OPL18	ACCACCCACC	10.0	6.0	60
OPL2	ACTGAACGCC.	12.0	3.0	25
OPM13	GGTGGTCAAG	12.0	5.0	42
OPN16	AAGCGACCTG	11.0	10.0	91
OPN18	GGTGAGGTCA	12.0	3.0	25
OPN20	GGTGCTCCGT	10.0	5.0	50
OPO12	CAGTGCTGTG	14.0	11.0	79
OPO15	CCAAGCTGCC	15.0	10.0	67
OPO5	GTGTCTCAGG	12.0	10.0	83
OPO7	CAGCACTGAC	15.0	10.0	67
OPP6	GTGGGCTGAC	10.0	5.0	50
OPP7	ACACAGAGGG	10.0	5.0	50
OPQ5	CCGCGTCTTG	13.0	8.0	62
OPQ15	GGGTAACGTG	18.0	9.0	50
OPR1	TGCGGGTCCT	13.0	12.0	92
OPT7	GGCAGGCTGT	15.0	8.0	53
OPV7	ACAGGTGCGT	10.0	4.0	40
TOTAL		212.0	122.0	58

ranged from 200-2000 bp, with an average number of bands per primer of 12.4 and furthermore, polymorphic bands were observed between 500-1500 bp (Fig. 1). The total number of fragments generated was 212, of which 122 bands were polymorphic and 90 were monomorphic (57.54%). The total number of amplified polymorphic DNA bands ranged between 3 (primer OPN18) and 12 (primer OPR1), wherein maximum number of polymorphic bands (10) were obtained from primers OPN16, OPO12, OPO15, OPO5, OPO7 and OPR1 (Table 1). The polymorphism percentage ranged from 25% (primer OPL2) to as high as 92% (primer OPR1). Average polymorphism across all 35 accessions was found to be 58%.

Cluster analysis: The UPGMA dendrogram was drawn to visualize relationships among 35 robusta coffee accessions (Fig. 2). The cluster tree analysis showed that the accessions were broadly divided in to three major groups. Subsequently these three main groups further divided into various sub-groups according to similarity between them. However, ten exotic accessions were found to be divergent did not fall in one group, but distributed in sub-groups. Three main groups branched at linkage distance unit of 80 into two major clusters. First major cluster consisted of 4 sub-clusters, in which first sub-cluster had JAVA, MADAGASKAR, SAIGAN, DR_8 and DR_9 joined at linkage distance of 23 units. Subsequently 2nd, 3rd and 4th sub-clusters joined at linkage distance of 25, 24 and 23 units, respectively. However, exotic accessions such as MADAGASKAR and SAIGAN, IVARY cost (S. 3855) and IVARYCOST, UGANDA (s. 1979) and GUATEMALA (s. 1481), COSTARICA (s. 3389) and COSTARICA (s. 3400) have showed close relatedness and merged at linkage distance of 45 units. However at 4th sub-cluster only DR selections such as 10, 13, 14, 15, 16 and 17 had showed close genetic relatedness and merged at 23 units of linkage distance. The second major cluster consisted of two sub groups, one with two accessions namely, UGANDA (S.880) and DR_3 which were close to each other with genetic distance of 28 units and merged with second group namely DR_6, 7, 11 and 12. Third major cluster has two sub-groups, one sub-group with DR_18, 19 and 20 joined at genetic distance of 20 units and second sub-group consisted with SIB_4, SIB_7, ROBUSTA BOLD, ROBUSTA HIGH YIELD and DWARF CxR joined at 20 units of linkage distance, however both these subgroups merged at 25 units of genetic distance.

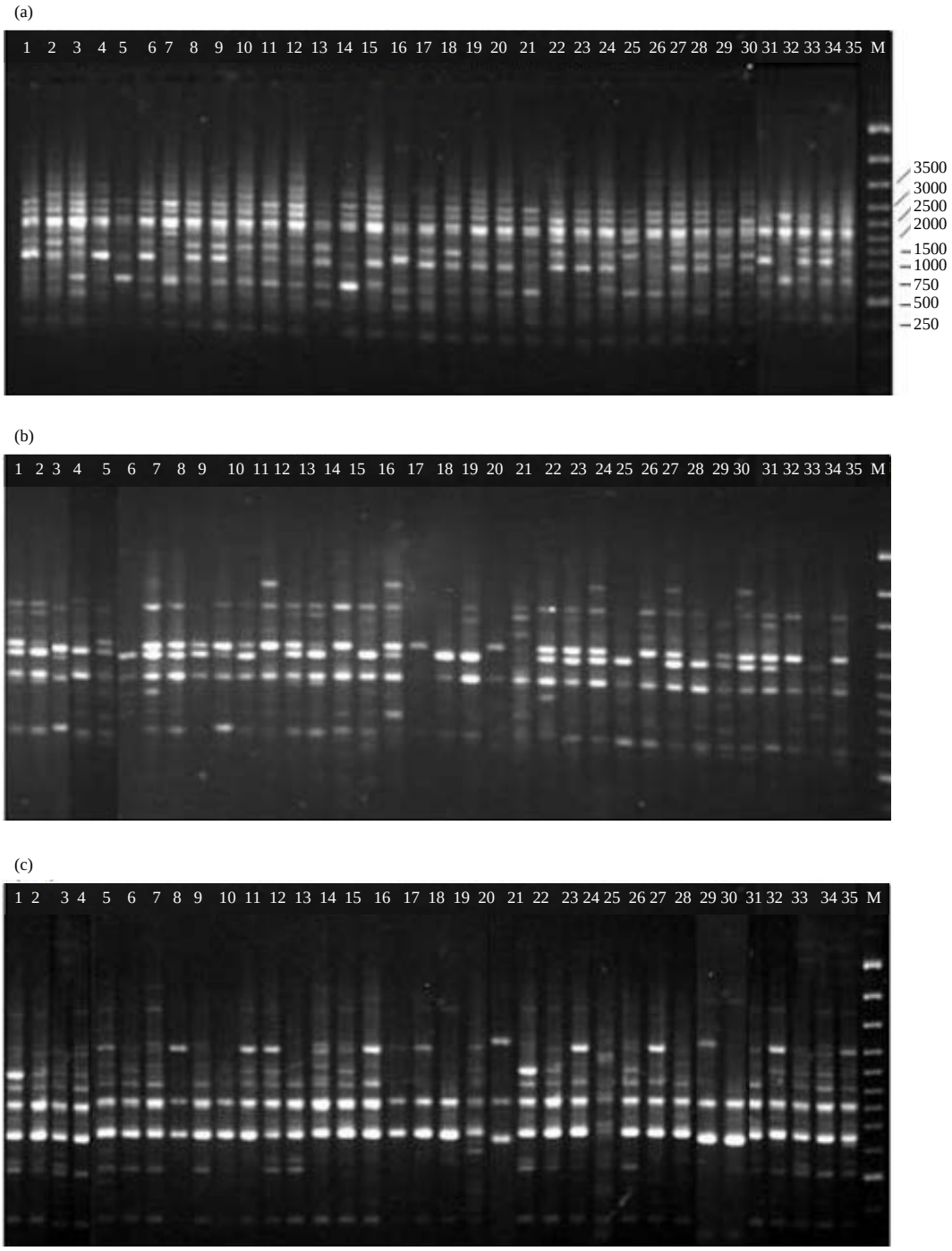

Fig. 1(a-c): RAPD gel profile of thirty five *Coffea canephora* accessions amplified with (a) OPP6, (b) OPQ5 and (c) OPO12 primers

Dissimilarity matrix: The dissimilarity matrix constructed by Squared Euclidean Distances (SED) which estimated all the pair wise differences in the amplification product as explained by previous study, revealed that the maximum dissimilarity of 31 units was observed between DR_8

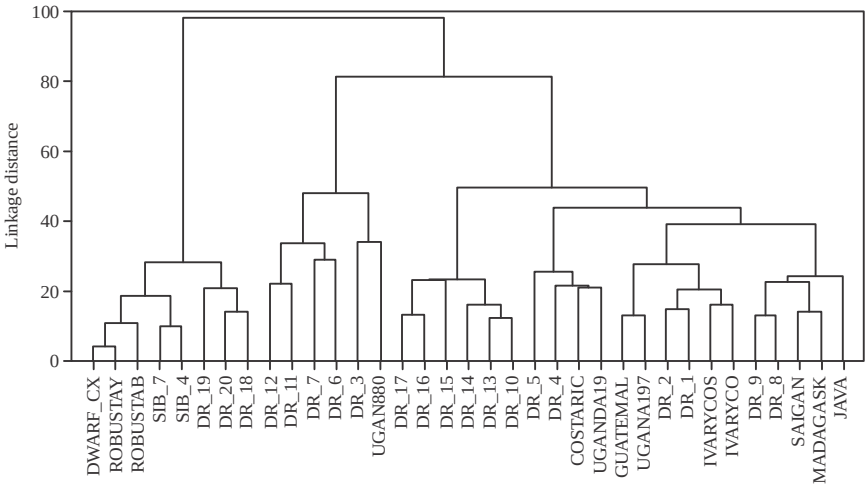

Fig. 2: UPGMA dendrogram showing the genetic relationships among the 35 *Coffea canephora* accessions

and DR_3, followed by 30 units between DR_9 and DR_4. The minimum dissimilarity of 2 units was observed between DWARF CxR and ROBUSTA HIGH YIELD accessions followed by ROBUSTA BOLD and robusta high yield with 5 units (Table 2). The clone DR_3 had more dissimilarity amongst the DR series from DR_4 to DR_20 (ranged from 19-28 units). Basically single clone did not obtain these DR accessions; however, they were collected during drought conditions in different estates of Wayanad district (Regional Coffee Research Station, Kerala State). Hence, these DR accessions not descended by single parent. In the dendrogram it was noticed that all the DR accessions were not grouped together, rather they were grouped with other accessions. With respect to exotic accessions there was not much dissimilarity was observed, except IVARYCOST (S. 3855) UGANDA (S.1979), GUATEMALA (S.1481) and IVARYCOST, wherein they showed 21,21, 23, 23 units, respectively with UGANDA 880.

Principal Component Analysis (PCA): Based on RAPD markers, three dimensional PCA formed three groups. The first group consists of exotic collections namely JAVA, MADAGASCAR, SAIGON, IVORYCOST, COSTARICA and GUATEMALA. The maximum accessions were grouped in second group contained 24 accessions with common variance. However, the third group had accessions like DR_3, 6, 7, 11 and UGANDA 880, showed unique variance which were placed distinctly in the analyzed PCA (Fig. 3).

Comparison of dendrogram and PCA generated by: A maximum linkage distance of 95 units was noticed in dendrogram based on RAPD data (Fig. 2). It revealed a genetically low variation among these accessions. Similar results were also noticed in the dissimilarity matrix constructed and PCA (Table 2 and Fig. 3).

A well dispersion of the points in the three-dimensional PCA based on RAPD markers showed very narrow genetic base and close distribution of the accessions. The correlation between dissimilarity matrices generated by these methods ($r = 0.3$) indicated they are positively associated but the relationship was non-significant. Consequently it requires large number of genetic markers

Table 2: Dissimilarity matrix constructed by Squared Euclidean distance using RAPD marker data

Codes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
JAVA	0																																		
MADAGSK	12	0																																	
SAIGAN	12	8	0																																
UGAN880	15	21	19	0																															
UGANDA19	13	12	11	17	0																														
COSTARIC	16	14	15	19	12	0																													
IVARYCO	15	15	15	21	17	12	0																												
UGANA197	12	13	12	21	14	13	10	0																											
GUATEMAL	17	12	14	23	14	11	15	8	0																										
IVARYCOS	14	16	15	23	18	15	9	10	11	0																									
DR_1	12	13	13	20	15	12	10	9	14	10	0																								
DR_2	13	14	12	22	17	15	11	10	15	12	9	0																							
DR_3	23	28	28	20	23	21	27	28	30	29	24	28	0																						
DR_4	17	17	15	19	12	13	16	15	17	16	15	16	19	0																					
DR_5	18	14	15	21	14	15	15	12	14	17	12	17	27	14	0																				
DR_6	18	22	22	21	18	20	21	21	22	21	20	20	18	16	18	0																			
DR_7	15	14	15	24	17	18	17	17	18	17	15	16	24	18	17	17	0																		
DR_8	14	12	12	24	15	17	15	11	13	11	12	14	31	18	17	24	16	0																	
DR_9	11	8	9	20	14	17	15	9	12	11	9	12	30	17	14	21	17	8	0																
DR_10	15	13	12	20	17	14	14	14	17	15	13	8	25	15	17	20	18	15	12	0															
DR_11	16	20	20	20	18	17	22	18	23	22	15	20	19	20	21	15	19	20	15	18	0														
DR_12	18	17	17	22	20	17	16	14	17	14	14	16	27	19	17	20	17	14	13	13	13	0													
DR_13	16	12	12	21	16	13	10	14	14	12	11	10	28	18	15	22	17	12	9	7	19	13	0												
DR_14	15	12	12	22	15	14	12	11	14	11	11	12	26	15	11	20	14	12	9	9	18	14	8	0											
DR_15	17	15	18	25	15	14	18	15	15	15	12	14	24	20	17	20	20	15	15	14	15	15	11	11	0										
DR_16	17	14	14	22	15	14	15	15	15	16	13	12	27	18	17	22	18	12	11	11	19	17	9	12	12	0									
DR_17	15	14	14	22	15	15	14	14	15	15	11	12	25	18	14	19	16	14	11	11	18	15	9	10	12	8	0								
DR_18	20	17	16	25	18	17	19	17	20	18	15	18	27	17	21	19	18	14	14	14	17	15	15	14	15	9	9	0							
DR_19	20	18	18	25	18	15	18	17	21	20	15	18	24	18	18	21	15	15	19	18	17	14	17	15	14	17	16	12	0						
DR_20	18	15	17	24	15	14	15	14	17	15	14	14	25	17	17	19	15	12	14	14	17	11	12	15	12	14	9	8	6	0					
SIB_4	22	18	20	28	21	17	18	18	19	18	17	19	28	23	21	24	17	15	18	17	20	14	16	16	13	14	11	11	10	9	0				
SIB_7	20	15	17	24	18	15	17	17	15	15	15	17	25	20	18	20	15	13	16	15	20	14	12	14	9	10	9	11	10	8	6	0			
ROBUSTAB	18	15	17	24	18	12	15	15	17	15	12	15	27	20	17	21	15	13	14	15	18	15	14	12	12	11	9	11	12	9	8	6	0		
ROBUSTAY	17	14	14	24	16	14	15	15	16	14	14	15	26	18	17	18	14	10	12	12	17	12	9	12	10	9	9	10	12	8	9	5	5	0	
DWARF_CX	17	14	15	24	17	14	17	17	16	15	15	16	26	20	17	18	13	11	13	13	17	12	12	12	11	11	11	12	12	10	10	7	5	2	0

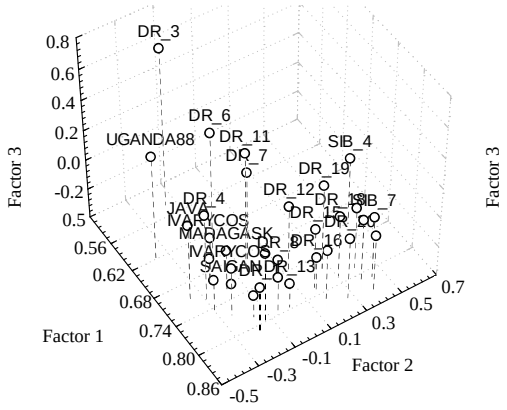

Fig. 3: Principle coordinate analysis of genetic distance among 35 *C. canephora* accessions

(RAPD) for significant correlation. It suggested requirement for much more number of markers as well as morphological traits to be used to distinguish various groups.

DISCUSSION

The DNA marker technologies, presently dominated by RAPD, AFLP, ISSR and SSR or microsatellites largely demonstrated in studying the genetic diversity of genotypes where it is one of the first basic steps in meaningful breeding programme (Karp *et al.,* 1997; Aremu, 2011).

In the present study assessed genetic diversity in 10 exotic and 25 indigenous *C. canephora* accessions clustered based on accessions allelic frequency than geographical origin. The collected 10 exotic robusta seedlings brought from respective countries, established at Central Coffee Research Institute, Balehonnur, Karnataka. However, DR_1 to DR_20 were collected during severe drought season of 1983, Regional Coffee Research Station, Chundale, where botanists conducted a survey and identified some good plants from various coffee estates of Wayanad, Kearla. They have been collected as open pollinated seeds from these plants and named as DROUGHT RESISTANT (DR) collections from 1-20. The other accessions like SIB_4, SIB_7, ROBUSTA BOLD, ROBUSTA HIGH YIELD and dwarf C x R were also estate collections identified from private estates of Wayanad.

It is well known that RAPD markers reveal polymorphism in coding and noncoding regions, as well as, repeated or single-copy sequences, covering the entire genome (Williams *et al*., 1990). Despite marker advantage and allogamous nature of the species, robusta accessions did not tend to show good amount of genetic variance in the present study. Since *C. canepora* is shallow rooted and drought sensitive, our objective was to find the genetic variance of better performing accessions performed under drought conditions. However in our study it is apparently found that among the 35 coffee accessions there is no significant genetic variation was observed and this was revealed by low level of polymorphic loci (58%). None the less, low polymorphism also did not contributed in reduction genetic diversity and therefore loss of alleles did not contributed genetic diversity (Lu and Bernardo, 2001). Furthermore, this indicates possibly accessions are genetically narrow and no trend in genetic drift (Masumbuko *et al*., 2003). Tshilenge *et al*. (2009) also indicated narrow genetic variation in robust accessions using ISSR markers (52%). it also suggested that the original clones that constituted the genetic basis of the populations studied were genetically narrow.

Interestingly it is noticed that exotic accessions were not showed much different from estate collections, indeed they are grouped with private estate collections. It revealed they are sharing genetic base and hence narrow genetic relatedness among them. However, the PCA analysis, it was observed that few DR collections namely DR_3, 6, 7 and 11 showed distinct variations with other accessions, which could be elite accessions for future breeding program. Similarly, it was observed with other DR collections, wherein they scattered in groups; perhaps the accessions are descended from few clones and little variation due to allogamous nature with modest out crossing. However, the other private estate collections SIB_4, SIB_7, ROBUSTA HIGH YIELD and ROBUSTA BOLD had with low dissimilarity grouped separately with DROUGHT RESISTANCE (DR) accessions. It is therefore concluded that despite the low level of genetic variation observed among the accessions, RAPD marker can be used as reliable technique for studying the specific variation.

CONCLUSION

To conclude, we could observe substantial genetic diversity using coffee accessions in spite of using a limited number of genotypes and markers. Although, this may invite criticism of insufficiency, the results obtained here have been nevertheless encouraging. However, a further study involving a larger group of diverse accessions and markers is required to underpin this argument. Since, the accessions included in this study are known to possess desirable attributes like abiotic factor drought tolerance, they qualify as suitable parental choice for breeding.

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