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Assessing Genetic Diversity using RAPD Molecular markers in *Coffea canephora* Pierre ex. Froehner (Robusta coffee): A Step towards Crop Improvement

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A B S T R A C T

The thirty one robusta accessions were analyzed for genetic diversity using RAPD molecular markers. Among the accessions, BR.11 indicated maximum dissimilarity with S.3325 (66 units) followed by S.3311 (61 units) and minimum dissimilarity was in BR.12 and L_1 valley robusta (9 units). Three major clusters were observed on the dendrogram with a maximum SED of 63 units among the accessions of first cluster, 58 units in second cluster and lowest of 46 units in third cluster. The first cluster accessions showed morpho-physiologically low root biomass, root to shoot ratio and moderately high total biomass. The second cluster accessions were with reasonably high root biomass and high root to shoot ratio. The third cluster accessions belong to high root and total biomass and water use efficiency types. The Principal Component Analysis (PCA) indicated genetic variation exists between the accessions. The accessions showed with over lapping and little tight clustering in three dimensional PCA revealed relatively low genetic diversity in Indian robusta gene pool. The contrast accessions i.e. S.3334 and L_1 valley robusta with respect to root, WUE and associated physiological traits were placed apart in PCAs and differed by 138 SED units and these accessions could be used to raise the mapping population in breeding programme.

Introduction

Coffee is one of the most widely consumed beverages in the world. Commercial production of coffee beans of two species, *Coffea arabica* L. and *Coffea canephora* Pierre ex Froehner, represents about 60 and 40% of the total coffee market, respectively. Brazil is the largest producer and exporter of coffee, contributing approximately 30% to the total production (ICO-International Coffee Organization, 2016). Genetic variations

among individuals of same or different species lead to genetic diversity, which must be ensured for sustainability of breeding programs and continued availability of agronomically superior cultivars.

Robusta coffee (*Coffea canephora*) is an allogamous and self-incompatible species native to central Africa. It was introduced to India from Java between 1900 and 1905 AD with the aim to combat leaf rust disease (Ram *et al.,* 1994). It manifests a high degree of rust resistance and highly productive at a relatively lower input level (Anon. 1985). In India, robusta coffee is predominantly grown in Karnataka, Kerala and Tamil Nadu states. Since, *Coffea canephora* has shown resistance towards stem borer, it is gradually replacing *Coffea arabica* in many parts. India is the third-largest producer and exporter of coffee in Asia, and the seventh-largest producer and fifth-largest exporter of coffee in the world. The coffee production in 2017-18 is estimated at 316,000 million tonnes (MT) and exports stood at 267,510 tonnes valued at US$ 652.35 million (Anonymous, 2017). However, the growth and productivity of coffee is constrained by the non-availability of water particularly during rain free periods (November to March/April). It is estimated that up to 56.74 % loss occurs mainly due to drought (Venkataramanan *et al.,* 2004). Therefore, developing drought tolerance robust coffee has become necessity. Assessing genetic diversity and relatedness among the genotypes is a critical step in any breeding and crop improvement programme. Thus, screening robusta coffee genotypes for drought tolerant traits is vital. Although several other techniques are available to screen drought tolerant genotypes in plants (Anon. 1985; Venkataramanan, 1985; Saraswathy *et al.,* 1992; Tounekti *et al.,* 2017), the molecular marker techniques (like RAPD, AFLP, etc.) are preferred in coffee (Ram and Sreenath, 2000; Awati, 2004; Bhat, 2002; Prakash *et al.,* 2005 and Silvestrini *et al.,* 2008). The RAPD technique has been useful in studying polymorphism, identifying genes of interest and characterizing genetic resources. RAPD markers are more preferred as the technique is simple, versatile, relatively inexpensive and able to detect minute differences (Williams *et al.,* 1990). In case of *Coffea* spp. RAPD technique has been used for different purposes like genetic diversity analysis of cultivated and wild accessions of *Coffea arabica* (Lashermes *et al.,* 1996). Anthony *et al.,* (2001) studied the genetic diversity among *C. arabica* accessions from spontaneous and sub spontaneous trees of Ethiopia.

The evaluation of genetic variability forms the basis for any crop improvement programme and its success depends on sufficient genetic variability among the genotypes to permit effective selection. Quantitative traits like root characteristics and physiological parameters are greatly influenced by environment, which reduces the progress and speed of selection programme. Hence, assessing the molecular diversity has greater relevance in tree crops with respect to breeding, selection and classification. Such information could be effectively used for selecting suitable parents for hybridization. Keeping this in view, the study was to look into the diverse robusta accessions using molecular markers for root traits, water use-efficiency morpho-physiological traits and relatedness among the 31 accessions of *C. canephora* accessions.

Materials and Methods

Most of the *Coffea canephora* accessions were obtained from the gene pool of Central Coffee research Institute (CCRI) farm, India. In addition, some of elite accessions were collected from the planters' field (Table 1). The leaf samples collected from each accession was cleaned and dried at 37 to 40 ^{o}C

for 48 hrs and ground to fine powder using a "Remi mixer". The DNA was then extracted using powder following modified CTAB method (Bhat *et al.*, 2002; Porebski *et al.*, 1997). The DNA was purified following phenol: chloroform (1:1 v/v) method and dissolved in TE (pH 8.0) buffer for PCR analysis. Quantification was carried out by using "Hoefers Dynaquant" (Pharmacia Biotech, USA) and the quality was verified by electrophoresis on a 0.8% agarose gel. DNA amplification by polymerase chain reaction was carried out in a volume of 25 µl, which comprised of 25 ng genomic DNA, 225 µM each dNTP, 1.5 mM $MgCl_2$, 1x buffer, 5 pmoles primer and one unit of Taq DNA polymerase (Bangalore Genie). The amplification was performed in a thermal cycler (PTC 100, MJ Research Inc.,). Total reaction consisted of 40 cycles each consisting three steps, denaturation at 94^{o}C for 1 min, annealing at 36^{o}C for 1 min and primer extension at 72^{o}C for 2 minutes with an initial denaturation step of 95^{o}C for 4 min and a final chain extension at 72^{o}C for 6 minutes. Amplified fragments were separated on 1.2% agarose (Gibco BRL) gels containing ethidium bromide (0.5 µg per ml gel solution) at 50 V for 5-6 h in 1x Tris Borate EDTA buffer (TBE). The gels were stained with 0.5 µg per ml of ethidium bromide and photographed under UV trans-illuminator and gels were documented by using Hero Lab Gel documentation system (Germany). PCR analysis was performed using 200 RAPD primers (decamer, Operon Technologies Inc. - K, L, M, N, O, P, Q, R, S and T series). Only 30 primers were selected based on number of bands produced (> 4 bands). Finally, 18 primers which yielded intense and clearly separating bands were selected for genetic diversity analysis (Table 2). The dendrogram was constructed by Ward's method of clustering using minimum variance algorithm (Ward, 1963).

Data analysis

Binary coding method was used to score the gels. By visual observation each fragment was given score of '0' (for absence) or '1' (for presence). The size of each fragment (molecular weight in base pairs) was estimated using 100 bp ladder marker, which was run along with the amplified products. Given amplified product (band) was designated as polymorphic, if it was found only in 28 (< 90 % of the total) accessions. By comparing all the possible pairs of accessions a distance matrix was developed (Sokal and Sneath, 1973). Dendrogram and principal component analysis (PCA) was performed using software STATISTICA.

Results and Discussion

The eighteen RAPD primers yielded a total of 256 bands in 31 robusta accessions. The number of bands per primer varied, primer OPN5 and OPP16 recorded maximum bands (20), whereas OPN20 yielded minimum bands (8). Overall, the average number of bands per primer was 14.2 (Table 3). Out of 256 bands, only 157 bands were found polymorphic (61.3%). The amplification product profile produced by primer OPN5 is shown in Figure 1.

Distance matrix

The dissimilarity matrix constructed by using Squared Euclidean distance (SED) revealed that, BR.11 with S. 3325 was 66 units of dissimilar followed by dissimilar with S.3311 (61 units), C x R (60 units), S.3318 and S.3347 with 59 units. Among the BR (*Balehonnur robusta*) series, BR.11 distinctly dissimilarity with other accessions because of its least compatible with others as female parent than a pollinator might be responsible for genetic purity of the line (Sreenivasan and Vishveshwara, 1981). In the present study,

BR.11 and S.3316 a descendant family of S.274 showed maximum dissimilarity with offspring's of S.270 robusta accessions. However, minimum dissimilarity was observed between BR.12 and L_1 valley robusta (9 units) followed by S.3347 and Tetraploid, Village robusta and S.3334, S.3332 and C x R with 13, 14 and 15 units respectively. The minimum dissimilarity between BR.12 and L_1 Valley robusta may be due to origin of both accessions from the seed progenies of S.275. Similarly, the clones or accessions i.e., S.3347, Tetraploid robusta, S.3327, S.4040, S.3318 raised from S.274 family showed a close similarity with each other in dissimilarity matrix constructed by SED using RAPD markers (Table 4).

Dendrogram

Cluster analysis revealed maximum SED of 138 units (Figure 2) on the dendrogram. Three major clusters were observed on the dendrogram. First cluster accessions were morpho-physiologically low root biomass, root to shoot ratio, instantaneous water use efficiency (WUE) i.e., Pn/g_s and Pn/E types (Awati, 2004). The small group of second cluster accessions was coming under moderately high root biomass, low shoot and high root to shoot ratio and net assimilation rate types.

The third cluster included the accessions belong to the group of high root biomass and high total biomass types with moderately high root to shoot ratio and shoot to root ratio and high WUE ones compared to first and second group of accessions.

A maximum SED of 63 units was observed among the accessions of first cluster, 58 units in second cluster and the lowest of 46 units in third cluster. The genetic diversity between first to second and second to third clusters was 96 and 138 units of Squared Euclidian distance respectively (Figure 2). In the present study, the existed genetic base of the cultivated robusta accessions at CCRI farm is rather narrow mainly because of many accessions were originated by a single cross of S.274 and S.270. Initially, S.270 and S.274 robusta selections were derived by mass selection from the indigenous eight superior mother plants of CCRI gene bank. Among the progeny of S.270 and S.274, seventeen mother plants were identified to be superior to that of parents (Vishveshwara, 1975) and the clones collected from identified seventeen mother plants were named as Balehonnur robustas (BR series) (Srinivasan and Vishveshwara, 1980). Prakash *et al.,* (2005) reported that majority of commercially cultivated forms of robusta coffee in India represent the selections from the descendants of initial introductions and revealed the higher amount of diversity present in the core samples than in Indian gene pool. Similarly in Arabica coffee, low genetic diversity was revealed by the analysis of RAPD markers (Bhat, 2002).

Principal component analysis

The PCA revealed similar clustering of 31 accessions as in the dendrogram. The three and two-dimensional PCA indicated that genetic variation exists between the accessions. Some of the accessions showed overlapping and little tight clustering of these accessions in three-dimensional PCA revealing a relatively low genetic diversity. Even though there is close clustering in the overall view, we could see some dispersion of the accessions on the two dimensional PCA indicating existence of variation. Some of the accessions like BR.11, BR.9 and S.3316 were placed distinctly in the analyzed PCA's. As a cross pollinated species, variation within the clones (BR.11 and BR.9 and S.3316), which were raised from S.274 and S.270 lines could be due to changes in genetic makeup of the accessions. However, the contrasting (S.3334

and L_1 valley robusta) parents used to raise the mapping population with respect to root and total biomass, root to shoot ratio, net assimilation rate, mean transpiration rate and instantaneous WUE were placed apart in both the PCAs (Figure 3 and 4).

Table.1 Robusta coffee (*Coffea canephora*) accessions used for analysis

Sl. No.*	Accessions	Details of Parentage
1	S.3317	S.274 ½ X S.270 $^{2/11}$
2	Kagnalla robusta	Elite material from Kagnalla Estate
3	S.4041	BR.10 X BR.9
4	S.3309	S.2361 $^{12/2}$
5	S.4048	BR.10 X BR.11
6	S.4046	BR.9 X BR.5
7	S.4045	BR.4 X BR.9
8	BR.12	S.275 X S.275 $^{3/9}$
9	L_1 valley robusta	*Coffea canephora* (Robusta coffee)
10	S.3349	S.270 $^{2/11}$ Self
11	S.4040	BR.9 X BR.10
12	S.3332	BR.14 $^{13/15}$ X BR.8 $^{11/7}$
13	S.3327	BR.1 $^{14/13}$ X S.274 $^{11/16}$
14	S.3329	BR.1 $^{14/13}$ X BR.9 $^{14/15}$
15	S.3322	S.270 $^{2/11}$ Bulk
16	C x R	*C.congensis x C.canephora*
17	S.3325	BR.1 $^{14/13}$ X BR.1 $^{10/15}$
18	S.3330	BR.1 $^{14/15}$ X S.274 $^{17/1}$
19	S.3347	BR.9 $^{14/15}$ X BR.1 $^{14/13}$
20	Tetraploid robusta	S.274 Seed treated with Colchicine
21	S.3318	S.274 $^{1/2}$ X S.274 $^{3/13}$
22	S.274	Released Selection of Coffee Board
23	S.4042	BR.9 X BR.11
24	S.3311	S.2361 $^{12/4}$
25	S.3339	BR.1 $^{21/15}$ X BR.12 $^{15/8}$
26	Village robusta	S.275 Seed progeny
27	S.3334	BR.8 $^{11/7}$ X BR.11 $^{9/15}$
28	S.4044	BR.9 X BR.4
29	S.3316	S.270 $^{2/11}$ X S.274 $^{1/2}$
30	BR.9	S.274 Clones
31	BR.11	S.274 Clones

*Serial numbers given in tables and gels represent respective *C. canephora* accessions throughout the study

Table.2 List of Primers with their sequences used in genetic diversity of robusta (*Coffea canephora*) 31 accessions

Sl. No	Primer	Sequence 5'- 3'	Total no. of bands	No. of Polymorphic bands
1	OPL18	ACCACCCACC	18	12
2	OPM13	GGTGGTCAAG	16	13
3	OPM16	GTAACCAGCC	16	11
4	OPN20	GGTGCTCCGT	8	4
5	OPN18	GGTGAGGTCA	14	10
6	OPN5	ACTGAACGCC	20	15
7	OPO12	CAGTGCTGTG	10	5
8	OPO7	CAGCACTGAC	16	9
9	OPP16	CCAAGCTGCC	20	9
10	OPP4	GTGTCTCAGG	11	5
11	OPP6	GTGGGCTGAC	12	7
12	OPQ15	GGGTAACGTG	16	11
13	OPQ5	CCGCGTCTTG	16	13
14	OPR1	TGCGGGTCCT	16	11
15	OPR12	ACAGGTGCGT	10	6
16	OPR3	ACACAGAGGG	12	5
17	OPN16	AAGCGACCTG	15	5
18	OPT7	GGCAGGCTGT	10	6
		TOTAL	**256**	**157**

Table.3 Summary table of the primers used, number of bands per primer and percentage of polymorphic bands

Total number of primers used	**18**
Average number of bands per primer	**14.2**
Number of polymorphic bands per primer	**8.7**
Percentage of polymorphic bands	**61.3**

Table.4 Dissimilarity matrix constructed by Squared Euclidean distance using RAPD data in robusta coffee

Ac No.	Robusta Accessions	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
1	**S.3317**	0	28	27	33	27	31	24	22	23	28	27	29	27	19	36	30	34	28	37	38	39	38	41	37	34	31	35	34	43	44	**56**
2	**Kagnalla**		0	31	23	25	25	22	30	33	38	33	37	37	39	40	44	44	36	51	54	47	36	33	43	40	47	45	38	53	42	**48**
3	**S.4041**			0	32	22	32	33	25	24	33	30	32	26	32	41	35	37	39	40	39	44	39	38	42	35	40	36	37	50	47	**53**
4	**S.3309**				0	24	22	27	27	32	37	28	30	34	38	31	37	39	39	40	47	42	39	30	36	37	40	38	35	54	37	**43**
5	**S.4048**					0	22	27	21	24	27	26	30	28	34	39	31	37	33	38	43	42	27	28	34	29	32	34	29	42	41	**43**
6	**S.4046**						0	21	23	24	31	28	32	28	40	35	37	43	35	42	39	44	37	28	40	37	36	38	25	50	37	**43**
7	**S.4045**							0	20	25	34	35	33	33	33	38	38	40	34	47	46	43	36	35	41	40	41	39	32	49	36	**36**
8	**BR.12**								0	9	24	25	19	17	29	28	20	26	28	31	32	33	32	31	29	34	25	27	26	49	38	**48**
9	**L_1 valley**									0	19	18	20	16	30	27	21	25	27	26	25	28	35	34	30	27	26	28	25	50	41	**53**
10	**S.3349**										0	27	25	25	27	26	24	30	30	29	36	29	38	37	33	38	33	37	28	53	46	**54**
11	**S.4040**											0	16	22	28	25	23	25	37	28	29	28	35	32	26	27	32	32	29	52	43	**57**
12	**S.3332**												0	20	26	21	15	23	35	26	27	24	35	34	24	29	24	26	31	52	43	**55**
13	**S.3327**													0	24	19	19	25	23	28	27	32	29	28	32	29	22	24	27	46	41	**49**
14	**S.3329**														0	29	23	33	33	36	41	38	35	44	34	37	30	34	35	46	49	**53**
15	**S.3322**															0	18	20	32	27	28	23	40	35	25	30	27	31	38	57	42	**56**
16	**CXR**																0	18	32	17	22	19	32	35	19	24	21	21	32	49	46	**60**
17	**S.3325**																	0	34	17	22	23	40	43	25	26	33	29	44	57	52	**66**
18	**S.3330**																		0	35	34	39	34	37	43	36	35	37	30	47	44	**46**
19	**S.3347**																			0	13	16	33	38	24	23	24	20	31	48	45	**59**
20	**Tetraploid**																				0	19	38	43	27	22	25	21	32	43	42	**56**
21	**S.3318**																					0	29	36	18	23	24	20	35	52	43	**59**
22	**S.274**																						0	17	23	30	29	29	34	35	40	**46**
23	**S.4042**																							0	26	35	32	34	27	44	35	**41**
24	**S.3311**																								0	23	28	24	39	46	45	**61**
25	**S.3339**																									0	25	17	34	41	40	**48**
26	**Village**																										0	14	23	40	35	**51**
27	**S.3334**																											0	25	36	35	**47**
28	**S.4044**																												0	41	26	**34**
29	**S.3316**																													0	43	**41**
30	**BR.9**																														0	**22**
31	BR.11																															**0**

Figure.1 A RAPD gel profile of 31 *C. canephora* accessions generated by the Primer OPN5

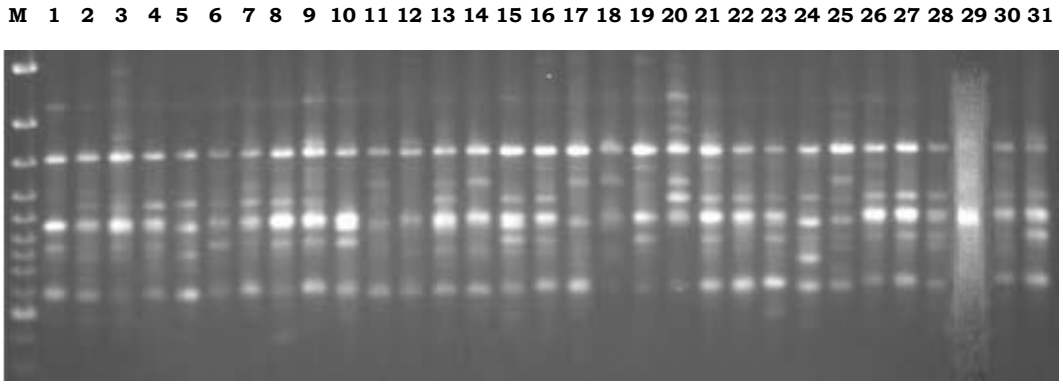


Figure.2 Dendrogram showing relationship among the 31 *Coffea canephora* accessions

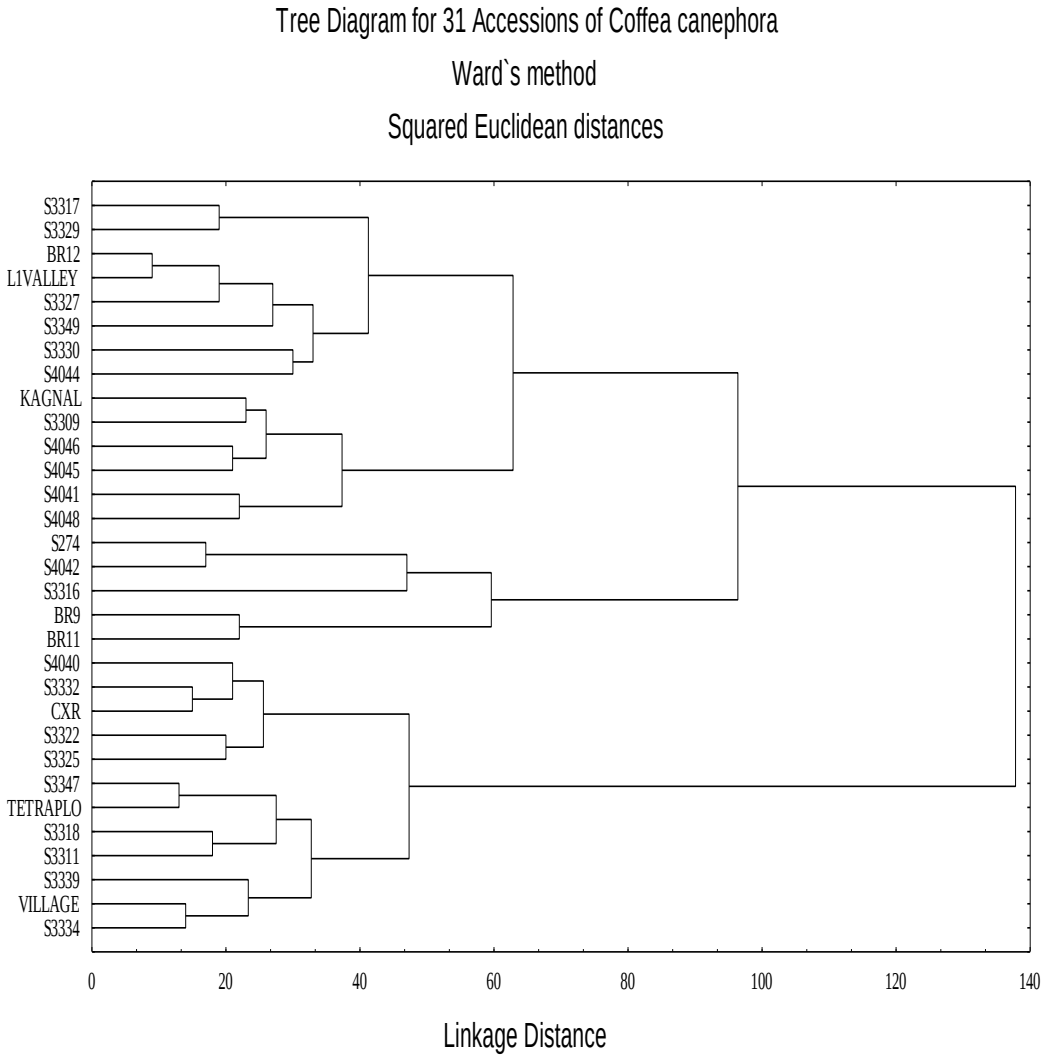


Figure.3 Three dimensional PCA showing the genetic relationship among the 31 *Coffea canephora* accessions

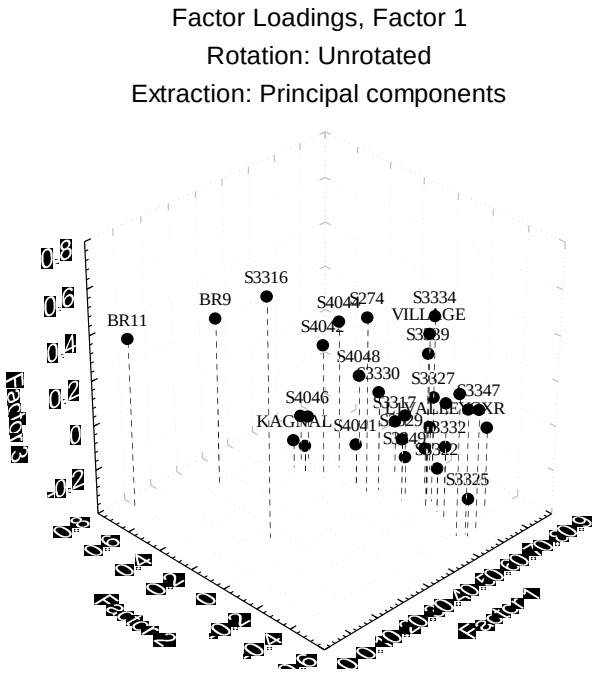
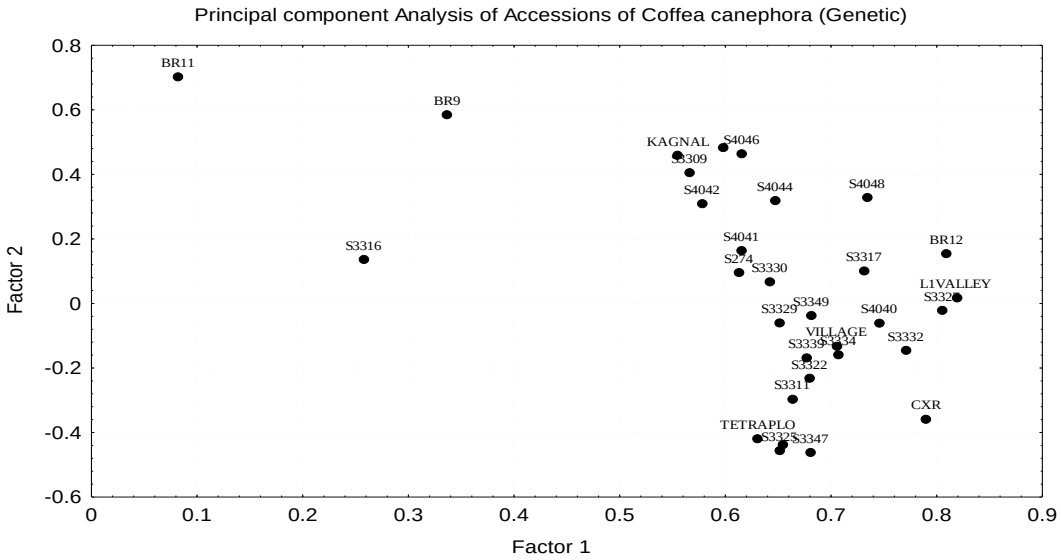


Figure.4 Two dimensional PCA showing the genetic relationship among the 31 *Coffea canephora* accessions



In spite of the fact that genetic diversity in *C. canephora* is low, in the present study RAPD markers could identify the differences among these accessions. For example, RAPD markers have been successfully employed to analyze the genetic diversity among the cultivated and sub-spontaneous accessions of *C. arabica* by Lashermes *et al.*, (1996) and

Anthony *et al.*, (2001). In *Coffea*, RAPD markers were used to identify polymorphism in different coffee accessions (Orozco-Castillo *et al.*, 1994; Lashermes *et al.*, 1996) and also to analyze spontaneous and sub spontaneous *C. arabica* trees form Ethiopia (Anthony *et al.*, 2001).

In conclusion, three and two dimensional PCA and clustering of accessions revealed that, relatively low genetic diversity in Indian robusta coffee. Among the accessions, BR.11 indicated maximum dissimilarity with S.3325 followed by a minimum dissimilarity in BR.12 and L_1 valley robusta. However, S.3334 and L_1 valley robusta were contrast with respect to root and total biomass, water use efficiency, root to shoot ratio, net assimilation rate, mean transpiration rate and also were placed apart in both PCAs and dendrogram and were used to raise the mapping population to study the association of markers and root traits in robusta coffee. The study confirmed the occurrence of traits diversity in robusta coffee accessions and this could be exploited in the genetic improvement of the crop through hybridization and selection. It is, however, necessary that use of more number of molecular markers with additional accessions need to be confirmed.

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