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MOLECULAR CHARACTERIZATION USING RAPD AND SSR MARKER IN DIVERSE POMEGRANATE [*PUNICA GRANATUM* (L.)] GERMPLASM

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ABSTRACT
Pomegranate (*Punica granatum* L.) belongs to the family Punicaceae exhibiting enormous morphological diversity is an important horticultural crop of region of western India. We have inferred evolutionary relationship of 50 diverse genotypes using RAPD and SSR markers. We have design new 14 SSR markers of which 3 were successful to depict polymorphic nature. Overall, 29 RAPD primers and eleven microsatellite (SSR) employed or molecular characterization and screening produced a total 80/99 and 54/66 DNA fragments RAPD and SSR marker respectively. Wider polymorphism become apparent with Average number of polymorphic bands per primer was found to be 5.7 (for RAPD) and 4.9 (for SSR). In SSR highest polymorphism (87.5%) was exhibited by PG5 while the lowest polymorphism (75.0%) was evinced with PG2, PG7 and PG31. The average polymorphism detected by the SSR loci in the present investigation was 80.9 %. UPGMA and NJ based analysis of the genotypes was performed, using Jaccard's similarity coefficient, PCA and PCO analysis. The highest and lowest similarities detected between genotypes were 1.0 and 0.31, respectively. Dendrogram in showed two major clusters with coefficient value r = 0.8147 showing the goodness of it of the dendrogram of RAPD. In SSR dendrogram showed two major clusters with co-efficient value r = 0.7627. RAPD and SSR markers showed to be a utilitarian instrument or analyzing the genetic variety of pomegranate.

KEY WORDS: (RAPD) random amplified polymorphic DNA and (SSR) simple sequence repeats markers.

INTRODUCTION

Pomegranate (*Punica granatum* L.) belongs to the family Punicaceae comprises single genus Punica and two sub-species *P. protopunica* Balf. (2n=2X=16) (Jalikop, 2010) and *P. granatum* L. (Smith, 1976, Stover and Mercure, 2007). It is the imperative delicious fruit crop of the tropical, subtropical region and commercially grown from Europe, Africa, Mediterranean region to the Himalayas terrain (north India) (Stover and Mercure, 2007, Ozgen, *et al.*, 2008, Morton and Miami, 1987) due to their nutrient rich pool of vitamin A, C & E with 15-19% sugar and other antioxidant (Chauhan, and Kanwar, 2012). India is a second largest producer 1.14 million tonnes (Chandra, *et al.*, 2010) after Iran. Out of this, nearly 80000 hectare area is covered in Maharashtra, which produces fruits of over 1 lakh metric tons (about 85% of the total production). In Gujarat 5.8 thousand hectare area is under cultivation with production 60.3 thousand tons fruit and productivity 10.4 tons per hectare in 2010-11 (Anonymous, 2011). In India major states that cultivate pomegranate are Maharashtra, Karnataka, Gujarat, and Andhra Pradesh and to a smaller extent in Rajasthan, Tamil Nadu and Himachal Pradesh. Pomegranate is often cross- pollinated crop (Jalikop and Kumar,1990) and exhibits five to six branch hermaphrodite flowers in different cultivars and ranged from 19.67 to 49% (Mir, *et al*., 2012) therefore it is evident with enormous morphological diversity. More than 250 germplasm lines are available in India. Total 187 (both exotic and indigenous) germplasm are available in national yield gene bank of National Research Centre on Pomegranate, Solapur. In the last 50 years, ten pomegranate cultivars have been recommended or only commercial cultivation. Bhagawa, Ganesh and Sinduri are popular varieties among farmers (Chandra, *et al.*, 2010). Pomegranate varieties *viz*., Ganesh, Bhagwa, Ruby, Arakta and Mridula are being cultivated in Maharashtra and Gujarat. So, current research was planned out to systematically analyze economically important variety with wider morphological variation at molecular level to unrevealed their ecological-evolutionary relationship.

Experimental Materials

For diversity study more than 2 years old, 50 accessions of Pomegranate were selected from Horticulture arm and AICRP Project on Arid Horticulture, S. D. Agricultural University. Includes variety Achikdana, Ahor Seedless, Bedana Suri, Bedana Sedana, Bassein Seedless, Damini, G R Pink, Kerala Collection, Jallore Seedless, Jodhapur Red, Jodhapur collection, Jyoti, Maha, Nimali, Sirin Anar, Saharanpur, Spendanadar, Surat Anar, Utkal, IC – 318705, IC – 318703, IC – 318779, IC – 318790, IC – 318753, IC – 318718, A K Anar, Bhagawa, Chawala, China Orange, Mridula.

Isolation of genomic DNA

Genomic DNA isolated, by talking about one g of fresh young disease free leaf materials, were ground to make a fine powder in liquid nitrogen and DNA was isolated by following the Cetyl trimethyl ammonium bromide (CTAB) method as described by Doyle and Doyle (Doyle

and Doyle, 1987) with some modification. The quality and concentration of the extracted DNA were estimated by spectrophotometer (Johnson, *et al*., 1955) and the samples were diluted to make a final concentration of 30-50 ngµl^{-1}.

RAPD and SSR Analysis and PCR amplification:

The 29 primers of RAPD (Table 1.1) and 24 SSR primers (Table 1.2) obtained from Bangalore Genie, Bangalore, India, was screened or the fifty pomegranates genomic DNA extracted or polymorphism observation. or RAPD, Polymerase chain reaction reactions were carried out in a 25 µl reaction volume containing 2.5 µl of PCR Buffer B (10X), 1.5 µl $MgCl_2$ (25 mM), 1 µl of each of dATP, dCTP, dGTP and dTTP, 0.2 µM of primer, 1 unit of Taq DNA polymerase, 50 ng of tempelate DNA. Amplifications were performed in a DNA thermocycler (Eppendorf, Hamburg, Germany), programmed or PCR, initial denaturation or 4.22 min at 94°C, followed by 45 consecutive cycles of 36 s at 94°C, 1.10 min at 36°C and 72°C or 1.10 min and final extension at 72°C or 5 min. likewise in **SSR** reactions except primers (forward and reverse) were used 0.2 µM of each, others were used in same conc. And the programmed as or initial denaturation or 4.22 min at 94°C, followed by 45 consecutive cycles of 36 s at 94°C, 2 min at 35-37°C and 72°C or 2 min and final extension at 72°C or 7 min (Bedaf *et al*., 2011). The annealing temperatures of the cycling parameter were readjusted or each microsatellite primers according to their calculated Tm based on the sequence composition: Tm =4° (G+C) + 2° (A+T)-3°C. The amplified products were subjected to electrophoresis in 1.5 % agarose gel in 0.4X TBE buffer [Tris borate (EDTA)] buffer running at 50 V or 2-2.5 h. The gel was stained with (4 µg/100 ml) Ethidium Bromide and viewed under UV light.

Data scoring

Data was scored or computer analysis on the basis of the presence or absence of the PCR products. The presence of the product / band was scored as '1' while the absence was designated as '0'. The data were maintained in the spreadsheet format or further analysis. Polymorphic Information Content (PIC) was calculated based on the frequency of alleles of each locus. $PIC_i = 1 - \sum P^2_{ij}$, Where, P_{ij} is the frequency of the j^{th} allele or the i^{th} marker locus and summation extends over n alleles. The polymorphism percentage was calculated as per lowing method: (Blair *et al*., 1999). Polymorphism (%) = {(Total number of bands - Number of monomorphic bands)/Total number of bands} x 100.

The cluster analysis and Neighbor Joining (NJ) clustering was done based on different similarity matrices using PAST version 2.17 software (PAleontological STatistics) (Hammer *et al*., 2001) and analyzed by the multivar tool with Jaccard's similarity coefficient(Jaccard, P., 1908), as well as or ordination plot based on Principal Component Analysis (PCA) and (PCO) Principal coordinate analysis were also carried out . Cophenetic correlation was determined to check the fitness of dendrograms obtained. Dendrogram was constructed using UPGMA (Unweighted Pair-Group Method with Arithmetic Averages).

RESULTS

Genetic diversity was studied on the basis of the banding pattern thus obtained by both RAPD and SSR primers clearly distinguished cultivars into different clusters showing sufficient diversity. The data collected from random amplification of polymorphic DNA with 14 arbitrary oligonucleotide primers produced a total 99 DNA fragments, among which 80 fragments were found to be polymorphic. As such the mean number of polymorphic bands per was found to be 5.7. The size of PCR amplified DNA fragment varied from 39.47 to 976.55 bp. The highest amplified band (12) was exhibited by primer OPYH18 and the lowest amplified band (4) was exhibited by primer OPAI18. The highest polymorphism (100.0%) was exhibited by primer OPY6 out of 8 band, all the 8 were found polymorphic while the lowest polymorphism (62.0 %) was evinced with OPAJ14. The average polymorphism detected by the RAPD loci in the present investigation was 80.3% (Table 1.4). The polymorphic information content (PIC) values ranged from 0.5882 to 0.9102 (average 0.7492), a reflection of allele diversity and frequency among the germplasm, were uniformly higher or all the RAPD loci tested.

The eleven microsatellite (SSR) produced a total 66 DNA fragments out of 24 primers, among which 54 fragments were found to be polymorphic. As such, the mean number of polymorphic bands per primer among fifty pomegranate genotypes was found to be 4.9. The size of PCR amplified DNA fragment varied from 35.7 to 943.05bp (Table 1.5). The highest amplified band (10) was exhibited by primer PG1 and the lowest amplified band (3) was exhibited by primer PG2, PG7 and PG31. The highest polymorphism (87.5%) was exhibited by PG5 while the lowest polymorphism (75.0%) was evinced with PG2, PG7 and PG31. The average polymorphism detected by the SSR loci in the present investigation was 80.9 %. PIC value or SSR primers ranged from 0.626 to 0.858 with an average of 0.7705. This value was good enough or efficient genetic analysis.

Construction of dendrogram or RAPD and SSR markers:

Preparation of dendrogram was done by using Jaccard similarity coefficients estimated similarity indices on the basis of 14 RAPD primers ranged from 0.31 (Guleshah Red, Dorsata and Kazil Anar) to 1.0 (between Kabuli, A. K. Anar and Nimali). Dendrogram clustered with the data generated by all primers and their amplicons grouped the 50 genotypes into two clusters in RAPD *i.e.*, Cluster A and Cluster B with coefficient value r=0. 8147. In RAPD cluster A was further divided into two clusters A1 and A2. The cluster A1 contained 5 genotypes and cluster A2 contained 2 genotypes. The cluster B was divided into B1 contained 6 genotypes and B2 divided in para cluster with all the rest groups containing genotype with Afghanistan origin. A and B phylogenetic study indicates that Kazil Anar was highly diversely grouped than all other genotypes under study as it showed a bifurcation pattern of dendrogram while genotypes Kabul, Basin Seedless and Saharanpur had highest similarity. from Out group study, Achikdana was found to be most out the group genotype as shown in fig. 1.2 while a strict pattern of evolution from

consensus tree analysis again indicated Achikdana and EC-4347 as the most diverse genotypes.

In SSR cluster (co-efficient value r= 0.7627) cluster A contained only one genotype china orange while cluster B was further divided into two clusters B1 and B2. The cluster B1 contained only one genotype Jodhpur collection and B2 group again divided into B2-a group contained 3 genotypes (Bosckalinsi, Ec-4347 and IC-318703) and B2-b further divided into two groups B2-b-1 contained Kabuli Yellow, Borekaunk, Chawala and Uttkal and B2-b-2 again diving in to B2-b-2-1contain genotypes Ahor seedless, Bassein seedless and Jallore seedless B2-b-2-2 para cluster contained all the rest genotypes with diverse origin. Again Achikdana was found to be most out the group genotype in NJ analysis (Fig. 1.6) while a strict pattern of evolution from consensus tree analysis again indicating Achikdana and Nimali were most diverse genotype.

Principle Component analysis

In the PCA plot (which includes two principal coordinates), it can be observed that Achikdana, Kazil Anar, Dorsata, Tabesta and IC- 318703 are placed farthest from Kabul in the 1st coordinate (X-axis), while Kabul, Kandhari, Bhagwa, Yercard and Nimali were placed farthest in the 2nd coordinate (Y-axis) (Fig. 1.3). China Orange, Ahor seedless varieties was located on the right side of the 2nd coordinate in the plot far from the EC-104348, and Guleshah Red from the PCoA (Principle Co-ordinate analysis) plot all the genotypes were distributed in major groups A and three different unique groups B, C and D as shown in fig 1.4. In this unique group B have Jodhpur Red Achikdana EC-4347 in the lower region of right side coordinates and group C have distributed in the center region of right coordinated (Dorsata, Kazil Anar and IC-318703) while only Tabetsa distributed in the upper region of D groups. Rest all genotypes are equally distributed in major group A around the left coordinates shown to be scattered in all the directions.

TABLE 1.1: List of RAPD primers

Sr. No.	RAPD Primer	Sequence (5'-3')	Sr. No.	RAPD Primer	Sequence (5'-3')
1.	OPYH18	GAATCGGCCA	16.	OPAG20	CTCCCAGGGT
2.	OPY6	AAGGCTCAAC	17.	OPAH16	TGCGCTCCTC
3.	OPY11	AGACGATGGG	18.	OPAH19	CAAGGTGGGT
4.	OPY13	GGGTCTCGGT	19.	OPAH2	GGCAGTTCTC
5.	OPBA3	GTGCGAGAAC	20.	OPAH20	CACTTCCGCT
6.	OPBB4	ACCAGGTCAC	21.	OPAI08	GGAAGGTGAG
7.	OPBB7	GAAGGCTGGG	22.	OPAI18	AAGCCCCCCA
8.	OPBB9	AGGCCGGTCA	23.	OPAJ08	TCGCGGAACC
9.	OPBD7	GAGCTGGTCC	24.	OPAJ14	GTGCTCCCTC
10.	OPBD17	GTTCGCTCCC	25.	OPAK19	ACCGATGCTG
11.	OPAD10	AAGAGGCCAG	26.	OPD17	TGATCCCTGG
12.	OPAD18	ACGAGAGGCA	27.	OPX19	TTTCCCACGG
13.	OPAE14	GAGAGGCTCC	28.	OPI-05	TGTTCCACGG
14.	OPAG08	GAGAGGCTCC	29.	OPI-18	AATGCGGGAG
15.	OPAG12	AAGAGCCCTC			

TABLE 1.2 : List of SSR primers used or molecular characterization

Sr. No.	SSR	Forward 5'-3'	Reverse 5'-3'
1.	PG 1	GGTCTGACTGGACCGTTGC	GAGAACGAAGATCCCGGTTT
2.	PG 2	CGGGATCTTCGTTCTCGAT	GGAATCCGTGAGCTGAGAGT
3.	PG 3	CGAAGAACGGCTAATCAACG	GATCCACCACGTCCAAC
4.	PG 4	CTGATGTAATGGCTGAGCAAA	GCACTTGAACAAAGAGAATGC
5.	PG 5	GCCACCTCTGCAATTCTCTC	GCAAAGGTTAGGCTCCGAAT
6.	PG 6	GGTTGCTCATCCCTTGACTC	GCGTCTGTCAGTGTCTTAGGC
7.	PG 7	CTTCCATTCCCCTAGCAACC	CTCCCCCGAACTTATCCTTC
8.	PG 8	CACCATAGACTTAAACGAGCACAA	GAAGCTCCATTGCCTCGTC
9.	PG 9	CTCCCTTCGGTTCTTGGTCT	ACGATGGAGTCCTTGTGGAT
10.	PG 10	CATCAGACTACGATGGCACT	GCATAATAGCCTTCAATTTACA
11	PG 21	CTACCGATTGAATGGTCCGGT	GATATGCTTAAACTCAGCGGGT
12	PG 22	CTACCGATTGAATGGTCCGGT	CCTGGGGTCGCGTTATTTGG
13	PG 23	CTACCGATTGAATGGTCCGGT	CGTTATTTGGATGGAGGCCAC
14	PG 26	GTCCGGTGAAGTGTTCGGAT	GATATGCTTAAACTCAGCGGGT
15	PG 27	GTCCGGTGAAGTGTTCGGAT	CCTGGGGTCGCGTTATTTGG
16	PG 28	GTCCGGTGAAGTGTTCGGAT	CGTTATTTGGATGGAGGCCAC
17	PG 31	ATCGCGGCGACGTGGGTGCTT	ATGGAGGCCACCGAGCAACG
18	PG 32	ATCGCGGCGACGTGGGTGCTT	CAACGCGGAAGGGACGGCTC
19	PG 33	ATCGCGGCGACGTGGGTGCTT	GCCCGAGGGGCCATGTACTG
20	PG 36	GTGGGTGCTTCGTCGCCGAC	CCTGGGGTCGCGTTATTTGG
21	PG 37	GTGGGTGCTTCGTCGCCGAC	ATGGAGGCCACCGAGCAACG
22	PG 38	GTGGGTGCTTCGTCGCCGAC	CAACGCGGAAGGGACGGCTC
23	PG 42	CGCGAGAAGTCCACTGAACC	CCTGGGGTCGCGTTATTTGG
24	PG 43	CGCGAGAAGTCCACTGAACC	CGTTATTTGGATGGAGGCCAC

For deciphering complexity of correlation between all genotypes with their molecular characterization based on Jaccard similarity distance analysis, it is apparent that similarity distance coefficient ranged from 0.27 (EC 1043448) to 1.00 (Nimali, AK Anar and Boscklansi). Based on similarity distance study, it indicated that distance between Kabul and Kandhari of same Afghan origin having maximum similarity of 0.94. However Kabul again, shows a distinct relationship with Nimali, AK Anar and Kabuli Yellow. In the SSR Principle Component analysis plot found that Jodhpur collection and China orange is placed far from Kabul present in the 1st coordinate (X-axis), while Tabesta and IC- 318703 were placed farthest in the lower first coordinate (Y-axis) (Fig. 1.7). Varieties with diverse origin located on the right side of 1st coordinate in the plot, including the Kazil anar, Gulshar Red and Nimali.

From the PCoA (Principle Co-ordinate analysis) plot all the genotypes were distributed in three major groups (A, B & C) and different unique group D (Fig 1.8). As shown in fig 1.8 unique group D is equally distributed around the right side of the coordinates 1 while group A were majorly scattered on coordinate 2 at upper region and remaining group B and C were shown to be scattered around the lower region of coordinate 1. Group B contained Boscklalinsi, Jodhpur collection and China orange while group C contained Ahor seedless Jalore seedless and Bassein seedless.

Therefore, this results confirmed the previous studies (Bedaf, *et al*., 2003, Sarkhosh *et al*., 2006, Ercisli, *et al*., 2007, Sheidai, *et al*., 2007, Zamani, *et al*., 2007, Durgac, *et al*., 2008, Kanwar, *et al*., 2008, Sheidai, *et al*., 2008, Narzary, *et al*., 2009, Ranade, *et al*., 2009, Sarkhosh, *et al*., 2009, Hasnaoui, *et al*., 2011[b], Kanwar, *et al*., 2010, Zamani, *et al*., 2010, Singh, *et al*., 2013, Soriano, *et al*., 2011) concluding RAPD and SSR as an effective technique to reveal genetic diversity among pomegranate accessions. In previous studies, 55 SSR markers had the average Polymorphism Information Content (PIC) value across all loci ranging from 0.09 - 0.71 with mean 0.37 (Curro, *et al*., 2010, Ebrahimi, *et al*., 2010, Hasnaoui, *et al*., 2010[a], Pirseyedi, *et al*., 2010, Basaki, *et al*., 2011, Ismail, *et* al., 2014, Orhan, *et* al., 2014).

TABLE 1.3 : Per cent polymorphisms revealed by RAPD analysis

Sr. No.	Primer name	Total Band	Polymorphic band	% Polymorphism	PIC value	Band Size range (bp)		
1.	OPYH18	12.0	10.0	83.0	0.910251	79.31	-	848.20
2.	OPY6	8.0	8.0	100.0	0.864839	104.11	-	900.00
3.	OPY11	5.0	4.0	80.0	0.78064	200.00	-	527.10
13.	OPAE14	7.0	6.0	85.7	0.835152	103.14	-	737.49
14.	OPAG08	5.0	4.0	80.0	0.785183	200.00	-	750.81
15.	OPAG12	7.0	5.0	71.4	0.82193	183.26	-	711.80
17.	OPAH16	7.0	6.0	85.7	0.823478	86.13	-	974.56
18.	OPAH19	7.0	6.0	85.7	0.848444	121.80	-	976.55
19.	OPAH2	6.0	5.0	83.3	0.561343	76.19	-	664.90
21.	OPAI08	7.0	6.0	85.7	0.832576	80.00	-	600.00
22.	OPAI18	4.0	3.0	75.0	0.722083	39.47	-	210.60
23.	OPAJ08	8.0	6.0	75.0	0.825208	102.30	-	383.40
24.	OPAJ14	8.0	5.0	62.5	0.588264	41.46	-	213.20
26.	OPD17	8.0	6.0	75.0	0.850779	91.00	-	369.98
		99.0	80.0	1128.0	11.05			
		7.1	5.7	80.5	78.93			

TABLE 1.4 : Per cent polymorphism revealed by SSR analysis

Sr. No.	Primer	Total band	Poly-morphic band	% Polymorphism	PIC value	Band size range (bp)		
1.	PG 1	10.0	8.0	80.0	0.85895	51.90	-	943.05
2.	PG 2	4.0	3.0	75.0	0.62643	35.71	-	300.00
3.	PG 3	5.0	4.0	80.0	0.75493	39.30	-	343.60
4.	PG 4	7.0	6.0	85.7	0.81815	41.18	-	196.63
5.	PG 5	8.0	7.0	87.5	0.84223	37.50	-	802.67
6.	PG 6	7.0	6.0	85.7	0.82193	35.70	-	913.90
7.	PG 7	4.0	3.0	75.0	0.68246	53.80	-	311.79
8.	PG 8	6.0	5.0	83.3	0.80561	80.50	-	274.83
9.	PG 21	5.0	4.0	80.0	0.71823	62.06	-	363.98
10.	PG 27	6.0	5.0	83.3	0.81588	54.96	-	489.74
11.	PG 31	4.0	3.0	75.0	0.73137	62.07	-	591.18
		66.0	54.0	890.5	8.47617			
		6.0	4.9	80.9	0.77056			

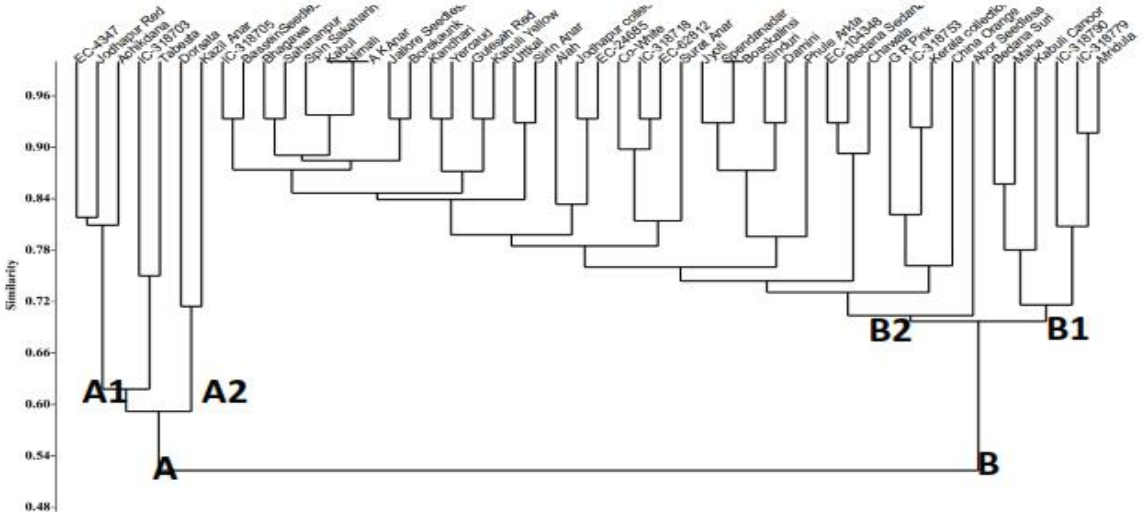

FIGURE 1.1: RAPD Dendrogram Clusters Analysis

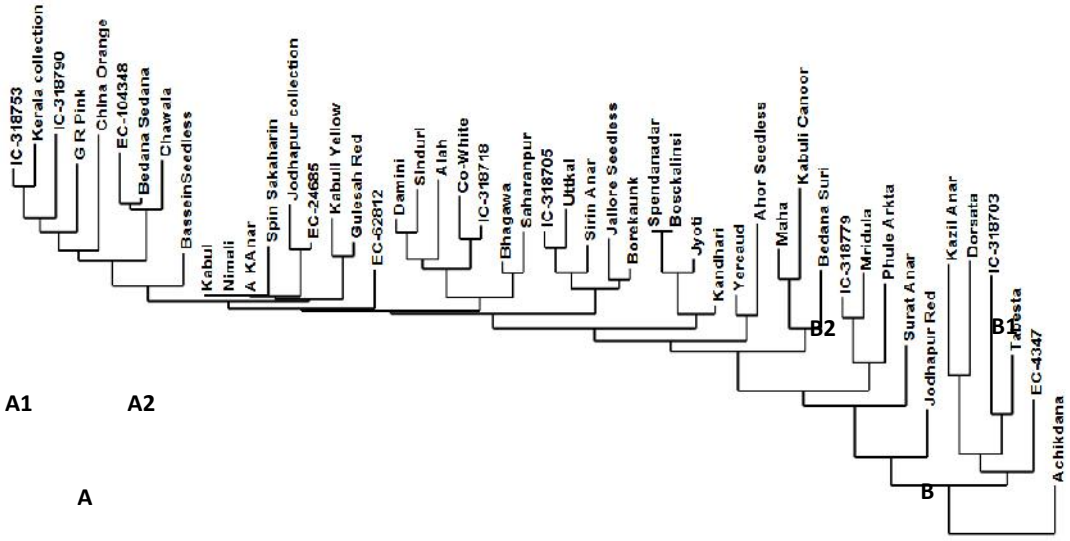

FIGURE 1.2 RAPD based (NJ) Neighbor joining phylogram or out-group analysis

FIGURE 1.3 RAPD PCA SCTTERED DIGRAM

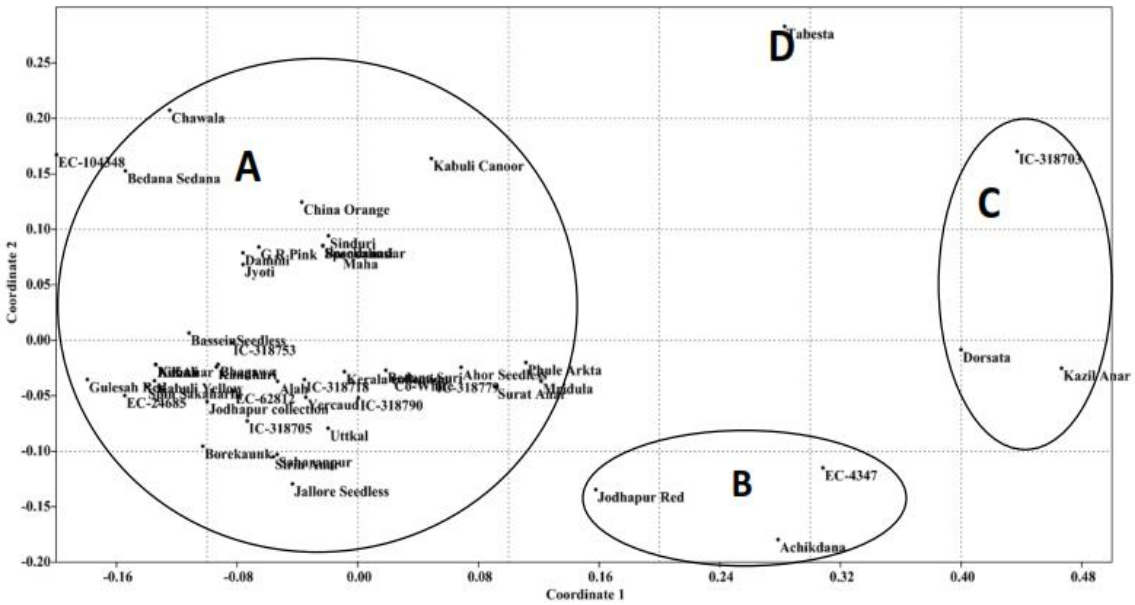

FIGURE 1.4 PCO Scattered Diagram

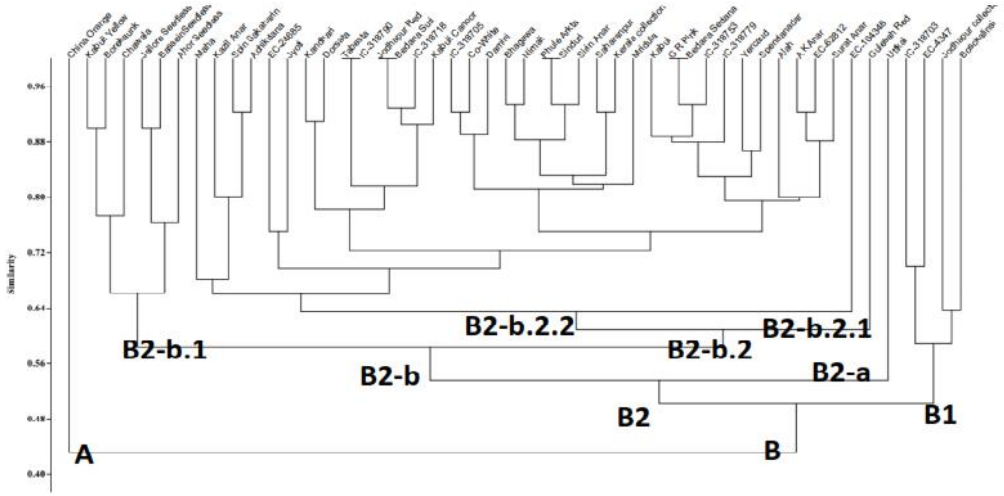

FIGURE 1.5 SSR Dendrogram Clusters analysis

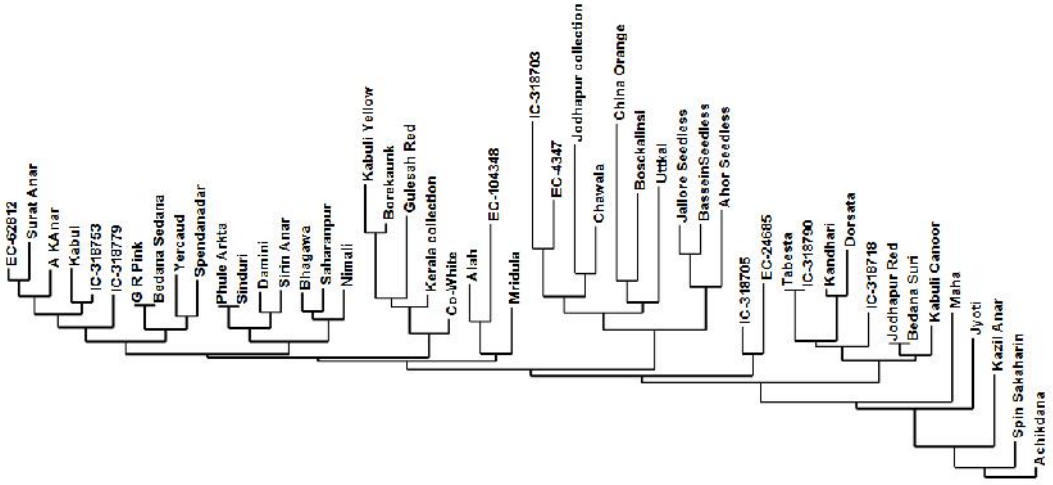

FIGURE 1.6 SSR (NJ) Neighbor joining analysis

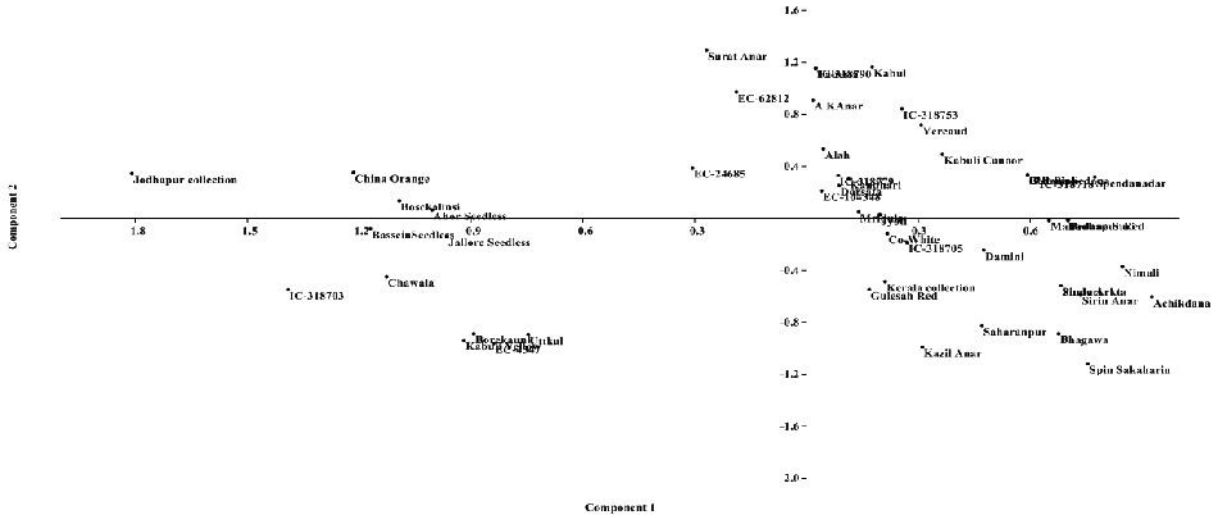

FIGURE 1.7 SSR PCA Analysis

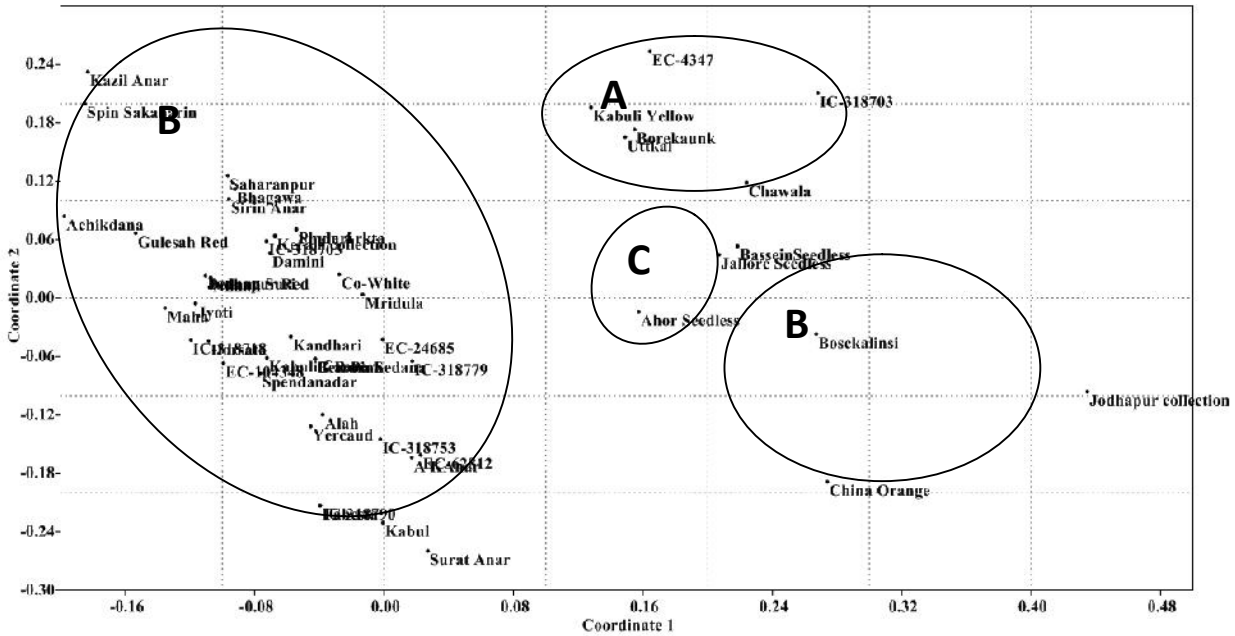

FIGURE 1.8 PCO Scatter diagram of SSR

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