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Genetic diversity assessment in mango ginger (*Curcuma amada* Roxb.) germplasm based on SSR and RAPD molecular markers

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Abstract
During the *kharif*-2018, the current study was conducted out in the molecular lab of the Department of Genetics and Plant Breeding, Navsari Agricultural University, Navsari (Gujarat). The experimental materials comprised of thirty different genotypes which were assessed with SSR and RAPD markers to assure the genotype divergence. The experiment's main goal was to see how much molecular variation there was in the available mango ginger germplasm, which will serve as base for future mango ginger improvement programmes. For molecular characterization, 9 SSR primer pairs were exploited in mango ginger accessions revealed all were monomorphic and hence the work was altered to RAPD. Furthermore, ten RAPD primers were tested and revealed a significant amount of polymorphism among the thirty mango ginger genotypes, with the best showing 100% polymorphism and high PIC values. The UPGMA algorithm was used to create a dendrogram based on the banding pattern of RAPD markers. The similarity coefficient can be anything between 0.43 and 1.00. The dendrogram clearly separated the thirty genotypes into two main clusters, each of which was further subdivided into eight sub-clusters, demonstrating the genotypic diversity of the genotypes investigated. The findings revealed a link between morphological features and the dendrogram produced by RAPD analysis.

Keywords: Dendrogram, genetic diversity, mango ginger, RAPD, SSR

Introduction
Curcuma is the most important genus in the Zingiberaceae family, with over 80 species of rhizomatous herbs adaptable to settings ranging from sea level to heights of 2000 metres in the Western Ghats and Himalayas (Sasikumar, 2005) [15]. Linnaeus established the genus name *Curcuma* in his *Species Plantarum* in 1753. The name is thought to come from the Arabic word *kurkum*, which indicates yellow (Salvi *et al*., 2002) [14]. This genus geographical range includes India, Thailand, Indochina, Malaysia, Indonesia, and Northern Australia (Apavajrut *et al*., 1999) [3].

Mango ginger (*Curcuma amada* Roxb., 2n = 42) is an important member of the genus *Curcuma* and is frequently known as mango ginger owing to morphological resemblance of its aromatic rhizome with ginger (*Zingiber officinale*) and smells raw unripe mango like aroma. The specific epithet amada comes from Bengali, which means mango ginger, and refers to the rhizome's unripe mango flavour. The crop is popular by many vernacular names like mango ginger in English, ama-haldi in Hindi, Amba Haldar in Gujarati, karpuraharidra in Sanskrit, amada in Bengali, mavinakayi in Kannad, mangaiinji in Tamil, mamidiallamu in Telugu and manga Inchi in Malayalam. The crop is found in wild as well as in cultivated forms and its distribution is confined to South-East Asia mainly India, Myanmar and Bangladesh. In India, it is under small scale cultivation in West Bengal, Gujarat, Uttar Pradesh, Kerala, Karnataka, Tamil Nadu and in the North-Eastern states. The crop evolved in the Indo-Malayan zone but is now extensively disseminated all over the tropics, from Asia to Africa, as well as in Australia (Sasikumar, 2005) [15]. *Curcuma amada* and *Curcuma zeodaria*, two of the ten *Curcuma* species, are found in both wild and cultivated forms throughout India. Many of the plants in this genus have medicinal, dyeing, and spice properties (Islam, 2004) [9].

In the Indian subcontinent, *Curcuma amada* has a long history of usage in folk medicine and as a culinary element among many ethnic groups. Salads, chutneys, and other value-added items are made using the rhizome. It's utilised in the creation of unique dishes, drinks, and medicines because of its exotic flavour and therapeutic properties and also used in cosmetic products (Nayak, 2002; Sasikumar, 2005) [13, 15].

The mango ginger rhizome is considered good as a stomachic because it has bitter, aromatic, cooling, astringent and carminative qualities (Anonymous, 1950) [2]. A rhizome paste has traditionally been used for healing of wounds, cuts and itching (Srivastava *et al*., 2006) [17]. The external use of the rhizome paste for sprains and skin diseases is also an old practice (Gupta *et al*., 1999) [6]. A decoction of rhizome (3ml) with common salt (2gm) is a good therapy for colds and coughs, while a whole plant paste with powdered long peppers is claimed to be effective for the treatment of piles (Kambaska, 2006) [11]. Combined with other medicines, rhizomes are also used to improve blood quality (Kapoor, 1990) [12]. It has a lengthy history of traditional applications that include everything from folk medicine to culinary preparations. Antibacterial, insecticidal, antifungal, and antioxidant activities are all present in this plant. The stomachic, anti-inflammatory rhizome is used to treat dyspepsia, headaches, and malaria. It's used to treat toothaches, stomach discomfort, and rheumatism by individuals from all over the world.

The genus has yet to undergo a full worldwide taxonomic review. Traditional taxonomic approaches, in combination with molecular biology technologies, may go a long way toward resolving the genus' taxonomic confusion. The development of DNA marker technology has offered an effective tool for plant genetic resource conservation and management. In the existing study, the use of molecular markers *viz*., Simple Sequence Repeats (SSR) and Random Amplified Polymorphic DNA (RAPD) bearing in mind that they produce highly polymorphic fingerprints. As a result, the investigation will use molecular markers to determine the genetic diversity and characterisation of mango ginger genotypes. Because they are not affected by the environment or developmental stage and can detect variation at the DNA level, molecular marker techniques may be able to overcome many of the limitations of morphological and biochemical markers for the discrimination of mango ginger accessions by providing genetic background for the observed phenotypic variability. DNA markers can be used to assess genetic variation among genotypes and to evaluate genetic drift in accessible germplasm (Jan *et al.*, 2012) [10].

Also, the genetic composition of *Curcuma amada* species needs to be assessed for efficient maintenance and conservation. There has been no previous report on the use of this method to elucidate the genetic diversity of *Curcuma amada* in various eco-geographical zones of Gujarat or India. The objective of this study was to evaluate the presence and pattern of genetic variability and relatedness among genotypes of mango ginger collected from selected areas of Gujarat using SSR and RAPD markers.

Materials and Method

1. Plant material

Thirty genotypes of mango ginger (*Curcuma amada* Roxb.) including check variety NVMG-7 (Chikhli local) planted at Research farm, Department of Genetics and Plant Breeding, Navsari Agricultural University, Navsari. Leaf samples from each of these genotypes were collected and were used for isolation of DNA.

2. DNA Extraction

Total genomic DNA was isolated from fresh leaves of 60-day-old plants using Doyel and Doyle's (1990) [5] modified Cetyl Trimethyl Ammonium Bromide (CTAB) technique. CTAB Extraction buffer (3%) 10 ml was made up of 1.0 ml of 1M Tris HCl (pH 8.0), 3.0 ml of 5M NaCl, 0.8 ml of 0.5 M EDTA (pH 8.0), 3.0 ml of% CTAB, and 2.1 ml of distilled water. Just before usage, 0.1 ml (1%) mercaptoethanol was added to a mixture.

3. PCR amplification

Table 1: Preparation of reaction mixture

Sr. No.	Reagents	Quantity	
		SSR	RAPD
1	PCR buffer (10X)	2.5 µl	2.5µl
2	Taq polymerase (5 U/µl)	0.3 µl	0.3µl
3	dNTPs mix	0.5 µl	2.0 µl (2.5mM each)
4	Primer (10 pM/µl)	1.0 µl (R & F each)	1.0 µl
5	Template DNA (50ng/µl)	1.0 µl	1.0 µl
6	Millipore water	18.7µl	18.2µl
Total		25.0 µl	25.0 µl

Table 2: PCR conditions

Sr. No	Steps	Temperature (°C)		Duration		Cycle
		SSR	RAPD	SSR	RAPD	
1	Initial denaturation	95	94	7 min	4 min	1
2	Denaturation	95	94	45 sec	1 min	35
3	Annealing	59	40	45 sec	1:20 min	
4	Extension	72	72	1 min	2 min	
5	Final extension	72	72	10 min	10 min	
6	Hold	4	4	--		∞

4. Electrophoresis of PCR amplified products

The amplified products of SSR and RAPD were analyzed using 2% and 1.5% Agarose gel, respectively. The bands were scored based on the molecular weight marker (100bp DNA ladder).

5. Data analysis

The NT-SYS-pc version 2.02e programme generated Jaccard's similarity coefficient by comparing genotypes pairwise based on the existence (1) or absence (0) of unique and shared polymorphic products. The unweighted pair group

technique using arithmetic averages was used to create a dendrogram using the similarity coefficient (UPGMA). The dendrogram was used in conjunction with Jaccard's similarity coefficient matrix to do a combined analysis. For self-pollinated species, the polymorphism information content (PIC) value reported by Botstein *et al*. (1980) [4] and updated by Anderson *et al*. (1993) [1] was computed as follows:

$$PIC_j = 1 - \sum_{i=1}^{n} P^2_{ij}$$

The frequency of the i^{th} allele for locus j is equal to P_{ij}. For this study, only data from polymorphic loci was used. For estimating the outcome, the procedures stated above were utilised. The (0) symbol was used to represent markers that did not amplify any allele.

Results and Discussion

In the present molecular diversity study, total 9 SSR primer pairs were exploited in mango ginger accessions to reveal polymorphism. In the research result, all were found monomorphic with banding pattern ranging maximum 1 per individual in all the loci and hence reported as 100% monomorphic (Fig. 2a). To further confirm the diversity, we used RAPD markers to reveal the molecular diversity among the studied mango ginger genotypes and the following results were obtained, which was summarized as under:

1. Polymorphism percentage and PIC value of RAPD primers

The result of molecular diversity with random primers revealed considerable amount of polymorphism among the studied genotypes with all primers. The amplification shown by different RAPD primers and polymorphism information content (PIC) of RAPD loci across various genotypes of mango ginger are presented in table 3.

Selected thirty genotypes of mango ginger were subjected to PCR amplification with randomly selected ten RAPD primers of different series which resulted in 94.87% of polymorphism (Table 3).

The total number of amplification products found were 39 with a maximum of 7 with the primer OPK-1 (Fig. 2b) and minimum of 2 with the OPE-10 and OPH-8 (Table 3). Out of ten primers eight primers *viz*., OPA-7 (Fig.2d), OPB-9, OPB-12 OPE-10, OPG-2, OPH-8, OPI-8 and OPK-10 exhibited 100 per cent polymorphism. The range of polymorphism varied between 80 to 100%. Whereas polymorphism percentage for primer OPK-1 and OPL-20 (Fig. 2c) were 85.71% and 80.00%, respectively and each produced one monomorphic band. The average number of bands per primer was 3.9. The highest numbers of band were produced by primer OPK-1(7) followed by OPA-7(5), OPB-12(5), OPL-20(5), whereas primer OPE-10 and OPH-8 both produced 2 bands which were lowest among all.

According to Akkaya and Buyukunal-Bal (2004), high Polymorphic Information Content (PIC) value can be attributed to the use of more informative markers. It is the reflection of allelic diversity and frequency among the genotypes. In the present study, PIC value ranges from 0.460 to 0.852. The highest PIC value observed for primer OPK-1 (0.852) followed by primer OPB-12 (0.800), whereas lowest PIC value was reported with primer OPH-8 (0.460). While PIC values for remaining primers were as follows, OPA-7 (0.693), OPB-9 (0.486), OPE-10 (0.500), OPG-2 (0.716), OPI-8 (0.653), OPK-10 (0.667) and OPL-20 (0.775).

Table 3: Polymorphism Information Content (PIC) of RAPD loci across various genotypes of mango ginger

Sr. No.	Primer	Sequence (5'-3')	Total number of band (a)	Total number polymorphic of band (b)	Monomorphic bands	Polymorphism% (b/a×100)	PIC value
1.	OPA-7	GAAACGGGTG	5	5	0	100	0.693
2.	OPB-9	TGGGGGACTC	3	3	0	100	0.486
3.	OPB-12	CCTTGACGCA	5	5	0	100	0.800
4.	OPE-10	CACCAGGTGA	2	2	0	100	0.500
5.	OPG-2	GGCACTGAGG	4	4	0	100	0.716
6.	OPH-8	GAAACACCCC	2	2	0	100	0.460
7.	OPI-8	TTTGCCCGGT	3	3	0	100	0.653
8.	OPK-1	CATTCGAGCC	7	6	1	85.71	0.852
9.	OPK-10	GTGCAACGTG	3	3	0	100	0.667
10.	OPL-20	TGGTGGACCA	5	4	1	80	0.775
Total			39	37	2	94.87	-

2. Dendrogram analysis based on Jaccard's similarity coefficient

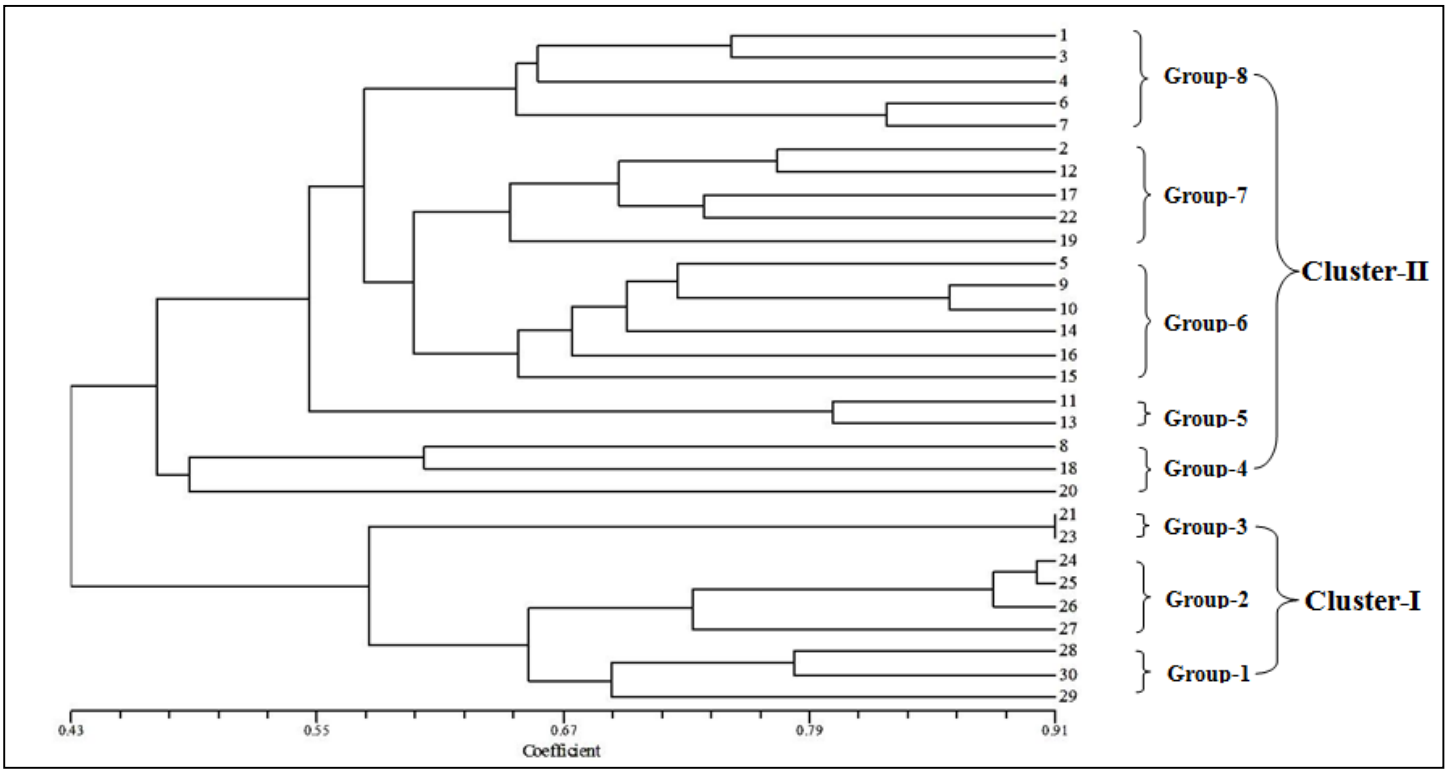
Dendrogram of exercised RAPD oligonucleotide categorized all studied mango ginger genotypes into genetically diverse clusters. The UPGMA dendrogram (Fig. 1) showed two main distinct clusters of mango ginger genotypes, which were also genetically diverse amongst themselves.

Cluster-I comprised of nine genotypes showing Jaccard's similarity coefficient value ranging from 0.55 to 0.91 (Fig.1) and was further divided into three sub-clusters. Sub cluster-I comprised of NVMG-28, NVMG-29 and NVMG-30, sub cluster-II consists of NVMG-24, NVMG-25, NVMG-26 and NVMG-27, while sub cluster-III contained NVMG-21 and NVMG-23. Jaccard's similarity coefficient revealed that high degree of similarity to the extent of 91% between the genotypes NVMG-21 and NVMG-23 in cluster-I at genetic level.

Cluster-II was the largest and it has included twenty-one of studied genotypes of mango ginger with similarity coefficients ranging amidst 0.29 to 0.75 (Table 4). It is interesting to note that the cluster-II is further divided into five sub clusters to simplify their comparative study. Sub cluster-IV includes three genotypes of mango ginger *viz*., NVMG-8, NVMG18 and NVMG-20. Sub cluster-V included only NVMG-11 and NVMG-13. Similarly, sub cluster-VI comprised of six genotypes *viz*., NVMG-5, NVMG-9, NVMG-10, NVMG-14, NVMG-15 and NVMG-16. Sub cluster-VII covered five genotypes *viz*., NVMG-2, NVMG-12, NVMG-17, NVMG-19 and NVMG-22. The sub cluster-VIII encompassed with five genotypes of mango ginger which were NVMG-1, NVMG-3 NVMG-4, NVMG-6 and NVMG-7.

Considering both, cluster-I and cluster-II the genotype NVMG-1 was observed distantly related to NVMG-28 and

NVMG-23 with similarity coefficient of 0.27 followed by NVMG-1 with NVMG-24 having similarity coefficients of 0.28 and NVMG-16 with NVMG-18, NVMG-8 with NVMG-19, NVMG-4 with NVMG-23 and with NVMG-28 with similarity coefficient of 0.29 (Table 4). High degree of similarity was found between genotypes. In cluster-I, NVMG-21 and NVMG-23 having similarity coefficients of 0.91 raced by NVMG-24 with NVMG-25 as well as NVMG-26 with similarity coefficient of 0.90. Similarly, in cluster-II, NVMG-12 was shown to be genetically linked to NVMG-2 and NVMG-10, with a similarity value of 0.77. According to the findings, RAPD's broad range of similarity coefficient values for linked genotypes gave better confidence in assessing genetic diversity and relationships among genotypes.



*Note: Vertical numbers from 1 to 30 represents genotypes NVMG-1 to NVMG-30

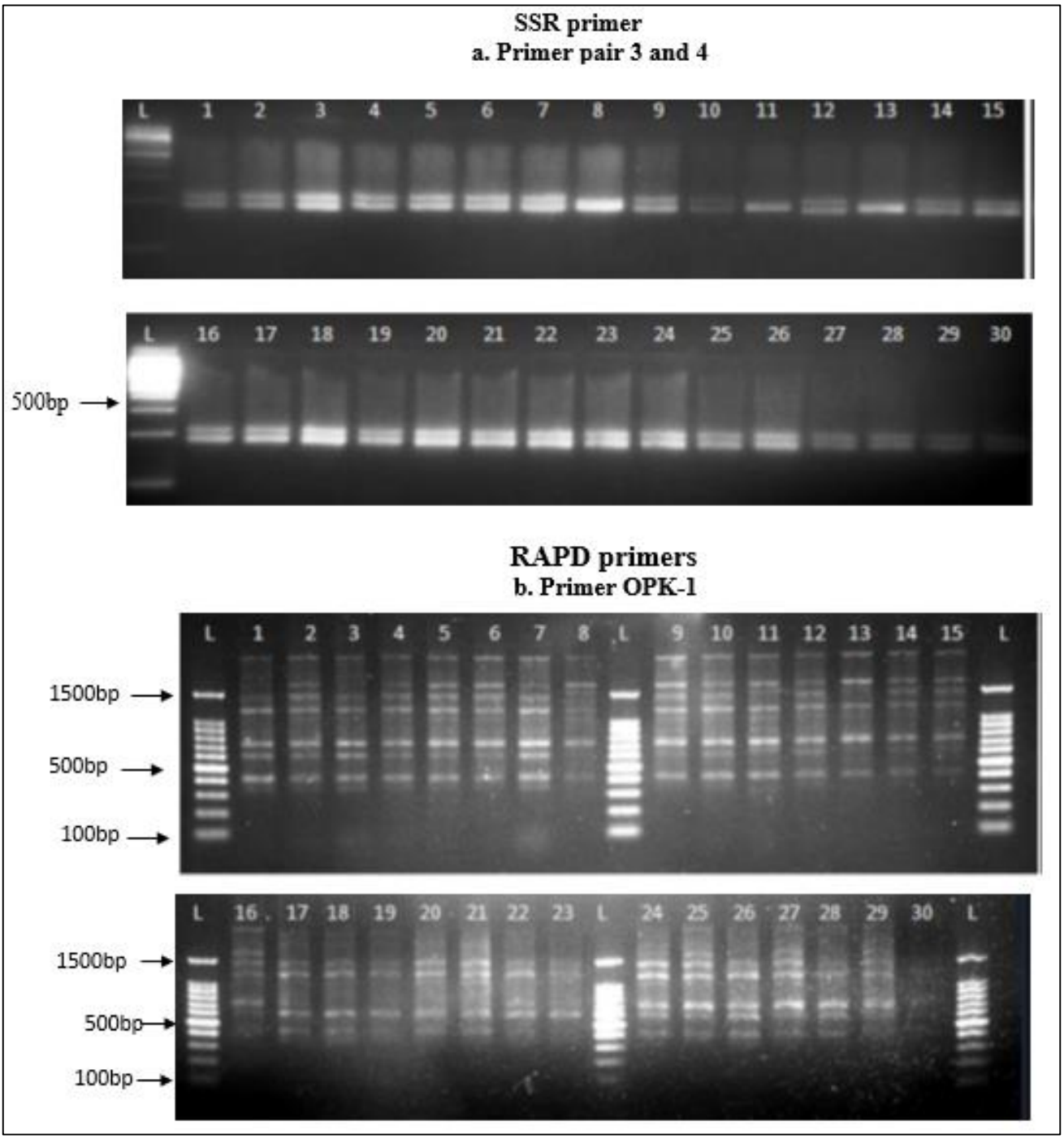
Fig 1: Cluster diagram based on RAPD data

Table 4: Jaccard's similarity coefficient for thirty genotypes of mango ginger based on RAPD data

	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
1	1.00																													
2	0.63	1.00																												
3	0.75	0.75	1.00																											
4	0.59	0.70	0.72	1.00																										
5	0.57	0.74	0.61	0.64	1.00																									
6	0.55	0.64	0.65	0.68	0.65	1.00																								
7	0.60	0.63	0.80	0.59	0.52	0.83	1.00																							
8	0.45	0.44	0.43	0.45	0.52	0.56	0.48	1.00																						
9	0.52	0.63	0.57	0.52	0.71	0.75	0.67	0.54	1.00																					
10	0.63	0.73	0.67	0.62	0.74	0.71	0.70	0.50	0.86	1.00																				
11	0.50	0.61	0.55	0.57	0.56	0.60	0.58	0.59	0.65	0.76	1.00																			
12	0.52	0.77	0.64	0.52	0.58	0.68	0.74	0.48	0.67	0.77	0.73	1.00																		
13	0.36	0.48	0.42	0.43	0.50	0.54	0.46	0.59	0.58	0.61	0.80	0.58	1.00																	
14	0.46	0.62	0.50	0.52	0.69	0.61	0.54	0.43	0.65	0.75	0.64	0.65	0.52	1.00																
15	0.50	0.61	0.42	0.43	0.56	0.67	0.58	0.46	0.73	0.68	0.64	0.73	0.50	0.64	1.00															
16	0.40	0.59	0.45	0.48	0.68	0.58	0.44	0.38	0.71	0.67	0.55	0.57	0.48	0.63	0.62	1.00														
17	0.65	0.75	0.68	0.55	0.61	0.58	0.64	0.43	0.64	0.75	0.62	0.71	0.48	0.56	0.55	0.52	1.00													
18	0.50	0.55	0.63	0.50	0.44	0.48	0.59	0.60	0.40	0.48	0.50	0.52	0.43	0.41	0.32	0.29	0.63	1.00												
19	0.37	0.65	0.50	0.45	0.46	0.44	0.48	0.29	0.42	0.50	0.45	0.62	0.39	0.48	0.45	0.50	0.67	0.53	1.00											
20	0.41	0.48	0.47	0.59	0.38	0.48	0.45	0.38	0.33	0.41	0.43	0.45	0.43	0.40	0.36	0.33	0.56	0.59	0.63	1.00										
21	0.32	0.43	0.42	0.33	0.45	0.39	0.41	0.36	0.46	0.48	0.44	0.46	0.44	0.47	0.39	0.42	0.54	0.44	0.46	0.43	1.00									
22	0.45	0.64	0.65	0.45	0.58	0.50	0.61	0.42	0.61	0.64	0.59	0.68	0.46	0.60	0.52	0.57	0.74	0.60	0.63	0.45	0.65	1.00								
23	0.27	0.43	0.42	0.29	0.40	0.34	0.41	0.31	0.41	0.43	0.39	0.46	0.39	0.42	0.34	0.42	0.48	0.44	0.52	0.38	0.91	0.65	1.00							
24	0.28	0.50	0.41	0.38	0.52	0.59	0.53	0.39	0.58	0.50	0.47	0.58	0.42	0.53	0.57	0.50	0.45	0.38	0.43	0.32	0.67	0.59	0.67	1.00						
25	0.33	0.52	0.42	0.39	0.58	0.56	0.50	0.36	0.60	0.52	0.44	0.55	0.39	0.55	0.59	0.57	0.42	0.34	0.40	0.29	0.63	0.55	0.63	0.90	1.00					
26	0.31	0.41	0.35	0.32	0.52	0.55	0.48	0.34	0.59	0.50	0.42	0.48	0.38	0.53	0.57	0.50	0.45	0.32	0.38	0.31	0.68	0.54	0.62	0.90	0.86	1.00				
27	0.31	0.50	0.35	0.41	0.42	0.55	0.48	0.43	0.53	0.45	0.47	0.53	0.42	0.40	0.57	0.40	0.40	0.32	0.38	0.31	0.47	0.39	0.47	0.77	0.74	0.68	1.00			

28	0.27	0.40	0.31	0.29	0.38	0.44	0.43	0.38	0.43	0.36	0.41	0.47	0.37	0.51	0.50	0.35	0.35	0.32	0.33	0.27	0.50	0.42	0.50	0.74	0.71	0.70	0.75	1.00		
29	0.32	0.50	0.36	0.33	0.43	0.42	0.36	0.31	0.40	0.41	0.42	0.48	0.42	0.53	0.42	0.45	0.41	0.29	0.43	0.32	0.52	0.39	0.56	0.61	0.63	0.53	0.62	0.74	1.00	
30	0.30	0.44	0.34	0.31	0.33	0.44	0.47	0.33	0.42	0.39	0.41	0.47	0.36	0.47	0.50	0.30	0.43	0.35	0.37	0.34	0.55	0.42	0.55	0.65	0.57	0.61	0.71	0.78	0.65	1.00

*Note: Vertical and horizontal numbers (bold form) from 1 to 30 represents genotypes NVMG-1 to NVMG-30. For example, stands for NVMG-7.



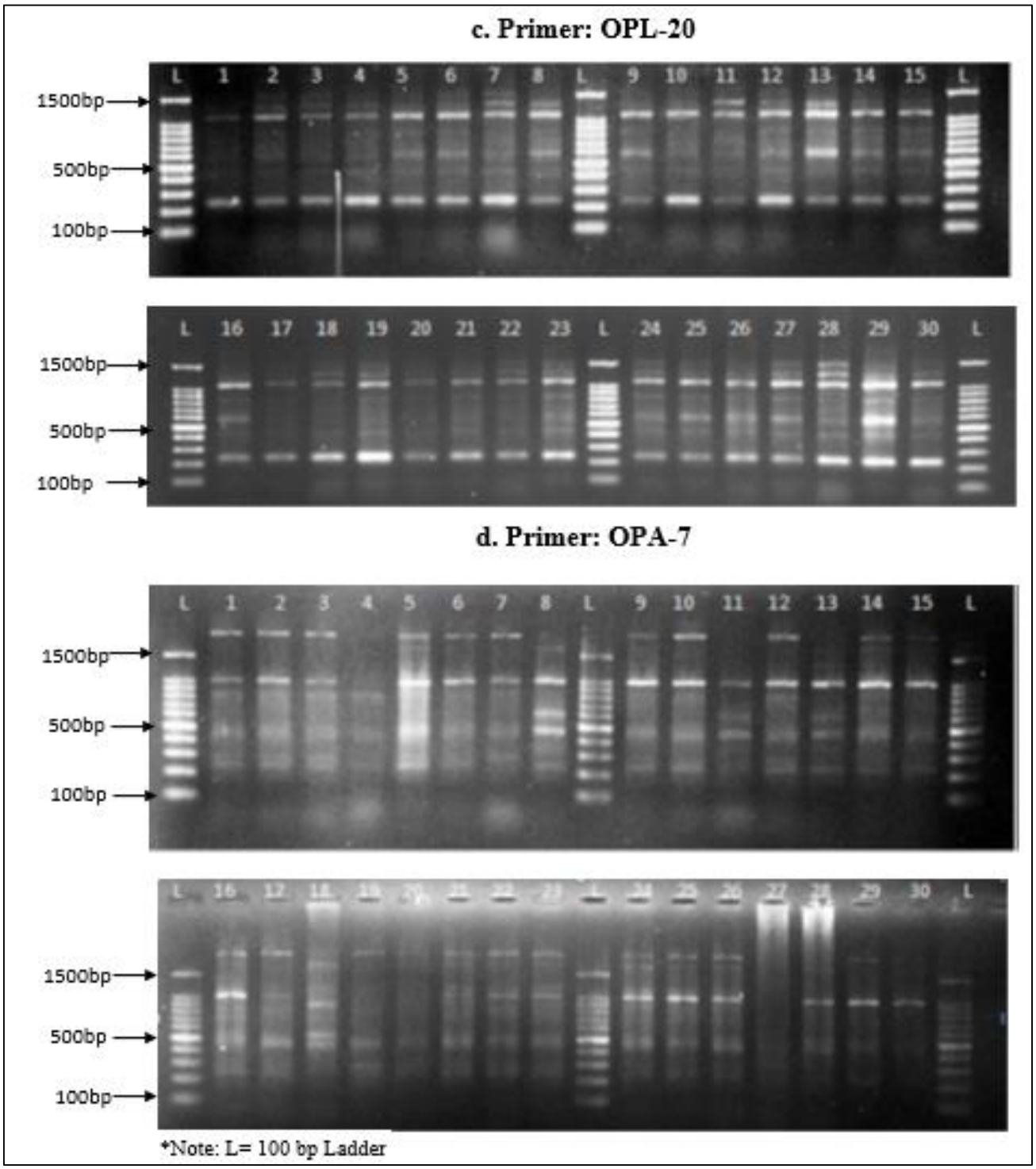


Fig 2: Agarose gel electrophoresis of PCR products of SSR and RAPD markers for thirty genotypes of Mango ginger

Conclusion

On the basis of the current study, it can be stated that RAPD genotype profiles can be utilised for diversification investigations due to their ease of detection and reliability. Furthermore, similarities in morphological traits within each group might be used to differentiate the dendrogram groups or clusters. Such a connection might be useful in developing a successful breeding programme. Similar kind of result was also reported in past studies by Syamkumar and Sasikumar (2007) [18]; Hikmat Ullah Jan *et al.* (2011) [7]; Singh *et al.* (2012) [16] and Ilyas *et al.* (2018) [8].

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