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DEVELOPMENT AND VALIDATION OF MOLECULAR MARKERS FOR FRESH SEED DORMANCY IN GROUNDNUT (*ARACHIS HYPOGAEA* L.)

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ABSTRACT

Groundnut (*Arachis hypogaea* L.) is a vital crop with significant agronomic value, yet its productivity is constrained by various environmental stresses. This study aimed to develop and validate SSR (simple sequence repeat) markers associated with seed dormancy and other key agronomic traits to facilitate groundnut breeding efforts. Using a diverse panel of 96 genotypes, 394 SSR markers were generated from target genomic regions associated with dormancy. Of these, 110 markers were validated, with 30 exhibiting polymorphisms suitable for genetic diversity analysis. Cluster and population structure analyses identified distinct genetic groupings, underscoring the potential of these markers to enhance breeding programs targeting yield and stress resilience in groundnut. This research contributes valuable molecular tools for advancing groundnut genetic improvement.

Key words : Groundnut, SSR markers, Genetic diversity, Marker validation, Breeding, Seed dormancy

Introduction

Groundnut (*Arachis hypogaea L.*), also known as peanut, is a globally significant legume crop cultivated primarily for its edible seeds, high-quality oil, and versatile by-products. It is the second most important cultivated legume, the fourth largest edible oilseed crop, and the third most significant source of vegetable protein worldwide (Savage and Keenan, 1994; Shilman *et al*., 2011). Groundnut plays an essential role in ensuring food security, improving nutritional health, and providing income for millions of smallholder farmers, particularly in tropical and subtropical regions. It serves a dual purpose as a food and an industrial crop. Groundnut kernels are consumed raw, roasted, or boiled, and processed for oil extraction, while by-products such as oil pressings, deoiled cakes, and dried haulms are widely used as animal feed and industrial raw materials.

India is one of the largest producers of groundnut, contributing significantly to global production. Among Indian states, Gujarat is the largest producer, accounting for 41% of the country's total production, followed by Andhra Pradesh and Tamil Nadu. Groundnut cultivation in India plays a crucial role in the livelihoods of rural farmers. According to USDA (2021), favorable climatic conditions have led to a 14% increase in India's oilseed production, with groundnut being a major contributor. However, despite its economic and agricultural importance, groundnut cultivation faces numerous challenges, including diseases, pests, drought, and pre-harvest sprouting. These challenges can significantly reduce yield and quality, posing a major threat to food security and farmer livelihoods.

The physiological state of groundnut seeds, particularly dormancy induction and germination initiation, plays a critical role in determining field establishment success following sowing (Nautiyal *et al.*, 2023). Seed dormancy and germination, while distinct phenomena, are essential for efficient crop management. Dormancy prevents pre-harvest sprouting (PHS) in moist conditions, while rapid germination ensures better field performance.

These processes are regulated by various physiological mechanisms and environmental factors (Koornneef *et al*., 2002). Groundnut germplasm exhibits significant variability in germination behavior (Bomireddy *et al*., 2024). Typically, bunch-type groundnuts lack dormancy and may sprout prematurely under moisture-rich conditions at maturity, whereas spreading and semi-spreading types demonstrate prolonged dormancy (Naganagoudar *et al*., 2016).

Spanish bunch varieties, characterized by a low degree of dormancy, are advantageous in preventing in-situ germination and PHS. However, extended dormancy, as seen in certain varieties, can delay normal germination, resulting in lower germination percentages in the field (Nautiyal *et al*., 2001). Addressing this challenge, growth regulators and chemicals have historically been employed to mitigate dormancy in cultivars (Rajan *et al*., 2020). However, breeding efforts are increasingly focused on developing cultivars with 14-21 days of fresh seed dormancy (FSD), striking a balance between PHS resistance and timely germination. This approach ensures resilience against rain-induced sprouting between maturity and harvest.

Despite the potential benefits, phenotypic selection for enhanced PHS resistance remains complex due to several factors. These include significant genetic and environmental interactions, variability in dormancy mechanisms among plant materials, and the polygenic nature of dormancy regulation (Yaw *et al*., 2008; Naganagoudar *et al*., 2016; Bomireddy *et al*., 2022; Zhang *et al*., 2022). Additionally, intergenic and epistatic interactions play a critical role in determining the genetic basis of dormancy (Khalfaoui, 1991; Bomireddy et al., 2022). Compounding the issue, controlled environments such as germinators and sprinkler rooms, while ideal for PHS phenotyping, are impractical for large-scale screening in breeding programs.

Recent advances in molecular biology and genomics have revolutionized groundnut breeding programs. Molecular markers such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs), and single nucleotide polymorphisms (SNPs) have been utilized for genetic mapping and trait identification in groundnut. Among these, simple sequence repeat (SSR) markers have proven particularly valuable due to their multi-allelic, co-dominant inheritance, high reproducibility, and ease of automation (Parida *et al*., 2009). SSR markers derived from expressed sequence tags (ESTs), known as genic SSRs, are especially advantageous as they target functionally relevant genomic regions, making them more predictive of agronomic traits.

Despite these advancements, groundnut breeding still faces significant challenges. The current number of available molecular markers, especially genic SSRs, is insufficient for the crop's large genome size and 20 linkage groups (Varshney *et al*., 2009). The lack of dense and informative genetic maps limits the efficiency of marker-assisted selection (MAS) for important traits such as seed dormancy, drought tolerance, and disease resistance. Furthermore, the Spanish and Valencia types' non-dormant nature makes them particularly unsuitable for regions prone to high rainfall during harvest, exacerbating yield losses.

To address these challenges, this study focuses on developing and validating novel SSR markers derived from EST sequences in groundnut. These markers target genes associated with critical agronomic traits, including fresh seed dormancy, drought tolerance, and yield potential. By mining publicly available EST databases, this research seeks to expand the repertoire of genic SSR markers for groundnut, enhancing the efficiency of molecular breeding programs. Additionally, the study aims to analyze the genetic diversity and population structure of groundnut genotypes to provide insights for developing resilient and high-yielding varieties.

This research holds promise for improving groundnut production by addressing the dual challenges of yield loss and environmental stress. The findings are expected to contribute significantly to sustainable agricultural practices and enhance the livelihoods of farmers dependent on groundnut cultivation.

Materials and Methods

Plant Materials

The study utilized 96 groundnut genotypes obtained from the ICAR-Directorate of Groundnut Research, representing a diverse range of genetic backgrounds, including both cultivated and wild relatives of groundnut.

DNA Extraction

Genomic DNA was extracted from leaves harvested from ten days old seedlings to each genotype by using the protocol described by Doyle and Doyle (1987). The quality and quantity of DNA were assessed using agarose gel electrophoresis and spectrophotometry.

SSR Marker Analysis

A total of 110 SSR markers were initially screened for polymorphism. Polymerase chain reaction (PCR) was

Table 1: Features of microsatellites identified by MISA.

	Genomic SSR from fresh seed dormancy linked QTL at A0909	Genomic SSR from fresh seed dormancy linked QTL at B05
Sequence location/Sequence examined	Aradu.A09: 114850050.115351249	Araip.B05: 114451556.116695578
Total number of identified SSRs	116	275
Total size of examined sequences (bp)	501200	2244023
Number of SSRs present in compound formation	12	83
Di-nucleotide repeats	38	197
Tri-nucleotide repeats	52	60
Tetra-nucleotide repeats	8	10
Penta-nucleotide repeats	2	7
Hexa-nucleotide repeats	16	1

performed in a 10 µL reaction volume containing 1.0µl of genomic DNA, 2.0 µl 5X taq buffer, 1.0 µl $MgCl_2$, 0.2µl dNTPs, 1.0µl of each primer, and 0.2 µl Taq DNA polymerase. Amplified products were analysed along with 50bp DNA ladder (fermentas) on 6% non-denaturing poly acrylamide gel (PAGE) running on 1x TBE buffer at constant power resistance of 225 volts for about 2.5-3.0 hr and stained with ethidium bromide (Benbouza *et al.*, 2006). The gels were documented in automated gel documentation system (Fujifilm FLA-5000).

Data Analysis

Molecular weight for each amplified allele was measured in base pair using Alpha-Ease FC 5.0 software. The size range of the amplified fragments for each microsatellite was estimated by using 50 bp DNA ladder and 100bp DNA ladder (Fermentas, USA).

The scoring data was used to analyse the diversity within the studied genotype and polymorphic information content using power marker V3.25 (Liu and Muse, 2005).

Phylogenetic tree was constructed using pair wise distance matrix computed by calculating a similarity matrix using a free tree (Pavlicek *et al.*, 1999). A nonweighted neighbour joining tree was constructed dissimilarity index. SSR amplicon obtained from each entry were resolved as a single band on the metaphor gel system and the data set were used to do the analysis. The genetic structure of the populations was also studied by using the Bayesian model-based approach proposed by Prichard *et al*., (2000) to assign the genotypes into genetically structured groups. The model assumes K number of populations characterized with a set of allele frequencies at each locus that are in Hardy-Weinberg equilibrium. The application tests the presence of a population structure (K > 1) and assigns the individuals from the sample population into groups for a given number of populations (K) in a way Hardy-Weinberg disequilibrium and linkage disequilibrium (LD) is maximally explained. The software package STRUCTURE version 2.3.4 (Prichard *et al.*, 2010) was used to perform this analysis. Optimum number of populations was inferred by running an admixture ancestry model with correlated allele frequencies starting from two populations K = 1 to K = 10, with 20 runs at each K. The ΔK shows a clear peak at the true value of K. This calculation was done by using an online software program called STRUCTURE HARVESTER, which is a python program with a web based front end for quickly parsing and summarising output data from STRUCTURE (Earl and volholdt, 2012). Inferred ancestry estimates of individuals (Q-matrix) were derived for the selected subpopulation (Pritchard *et al*., 2000).

Results and Discussion

Development of SSR marker

The genome sequence of groundnut, as identified by Kumar *et al*., (2019), was utilized for the development of SSR markers in this study. Two candidate genomic regions, spanning 2.4 Mb on the B05 pseudomolecule and 0.74 Mb on the A09 pseudomolecule, which are responsible for controlling fresh seed dormancy, were selected for marker development.

SSR markers were designed for di-, tri-, tetra-, penta-, and hexa-nucleotide motifs, as well as for compound microsatellites, using MISA software (Beier *et al*., 2017). Primer3 software (You *et al*., 2008) was employed to facilitate the design of these markers. A total of 116 SSR markers were developed from the A09 genome, while 275 SSR markers were developed from the B05 genome.

The detailed distribution of nucleotide motifs in both genomes is as follows:

- In the A09 genome, the SSR markers consisted of 12 di-, 38 tri-, 52 tetra-, 8 penta-, 2 hexa-nucleotides, and 16 compound microsatellites.
- In the B05 genome, the SSR markers consisted of 83 di-, 197 tri-, 60 tetra-, 10 penta-, 7 hexa-nucleotides, and 1 compound microsatellite.

These newly developed SSR markers form the basis

Table 2: List of 96 Groundnut genotypes used in the study.

S.	Genotype	S.	Genotype
1	JGN-3	**2**	VRI-4
3	JGN-23	**4**	JGN-24
5	TLG-45	**6**	GJG32
7	Co-1	**8**	LGN-1
9	Dharni	**10**	GJG-9
11	GJG-6	**12**	TMV-7
13	TMV-12	**14**	CO-1
15	ICG511	**16**	GG-3
17	JL220	**18**	TG-38A
19	ICGV-91114	**20**	VRI2
21	SB11	**22**	Pratap mughphali-1
23	ALR-2	**24**	Kisan
25	DH-3-30	**26**	AK-12-24
27	R-8808	**28**	TG-17
29	Jawan	**30**	Kadiri-4
31	Kadiri-9	**32**	Prasuna
33	GRG12	**34**	ICGV-86590
35	Tirupati-3	**36**	Kadiri-6
37	VRI-3	**38**	GG-7
39	ALR-3	**40**	Abhaya
41	JL24	**42**	Vemana(K-123)
43	MH-1	**44**	TAG-24
45	TGP-41	**46**	GG-2
47	JL-501	**48**	KRG-1
49	ALGO-06-320	**50**	DG38
51	Sapnish improved 1	**52**	TG-22
53	R-9251	**54**	DH-101
55	Jyoti	**56**	Girnar-3
57	Girnar-1	**58**	GJG-33
59	Narayani	**60**	GG-5
61	Kadiri-5	**62**	Dh-8
63	CO-3	**64**	GG11
65	G34	**66**	GPBD4
67	ICG(FDRS)-10	**68**	ICGS-36
69	AK-153	**70**	CO(Gn)-4
71	TKG-19A	**72**	TMV-2
73	G-2-52	**74**	ICGV-00350
75	S206	**76**	R-2001-2
77	TMV-9	**78**	OG-52-1
79	TG-26	**80**	Pratap mungphali
81	GG-8	**82**	Pratap mugphali-2
83	Kadiri Haritandhra(K-1319)	**84**	Tirupati-2
85	SG-84	**86**	JL286
87	Tirupati-4	**88**	JL286
89	R-2001-3	**90**	DH86
91	ICGS-1	**92**	JL776
93	GJG-31	**94**	RG-141
95	TG-51	**96**	VRI(GN)-6

for future genetic studies and breeding efforts aimed at improving seed dormancy traits in groundnut. The detailed features of SSR identification are presented in Table 1.

Validation of newly designed SSRs

Out of the 394 SSR primers developed, a subset of 110 primers was selected for validation based on their functional relevance. These primers were tested on a panel of 96 parental groundnut genotypes to assess polymorphism. The genotypes used for validation represent a diverse collection of cultivated groundnut lines, and the complete list of these 96 genotypes is provided in Table 2.

The polymorphism analysis revealed that out of the 110 SSR primers:

- 30 primers exhibited polymorphism.
- 53 primers were monomorphic.
- 27 primers failed to amplify.

This validation confirms the utility of these polymorphic SSR markers for distinguishing between groundnut genotypes, which is essential for marker-assisted selection and genetic diversity studies in breeding programs. Further details regarding the amplification sizes and polymorphic information content (PIC) values for these markers are presented in Table 3.

Among the 96 groundnut genotypes analyzed, eight genotypes exhibited the dormancy trait, namely Kadiri Haritandhra, DH-8, TMV-9, TG-37A, Co-1, TG-17, Tirupati-3, and TPG-41. These genotypes were identified based on their sequence homology with genes associated with fresh seed dormancy.

Several polymorphic SSR markers demonstrated high polymorphic information content (PIC) values, which indicates their utility in distinguishing dormant genotypes. Notable SSR markers with high PIC values include:

- DGR_D1 (0.8232),
- DGR_D23 (0.8195),
- DGR_D33 (0.7706),
- DGR_D62 (0.7445),
- DGR_D2 (0.7373),
- DGR_D15 (0.7239),
- DGR_D22 (0.7024).

These markers could be effectively validated on mapping populations and were able to differentiate the germplasm for dormancy. The 30 polymorphic SSR markers used in the study proved efficient in identifying genetic diversity related to seed dormancy.

The clustering of the dormant genotypes was as follows:

- Cluster I included Kadiri Haritandhra, DH-8, and TMV-9.
- Cluster II included TG-37A and Co-1.

Table 3: List of 110 primers validated on 96 groundnut genotypes.

S. No.	Primer Name	Sequence	Amplicon size range	M/P	PIC
1	DGR_D1	TTAGACCATCACATGCTTCCAC	310-449	P	0.8232
		GATGAGGTTGTTTGGTGCATTA			
2	DGR_D2	TCTTTCTCTTCCTCATCTTCAGC	173-229	P	0.7373
		CTTCTTGCACGTTCTTCTTCCT			
3	DGR_D3	AATGATGATGAGGTTTGGTTCC	397	M	-
		CCGCTTTGTAGTGTGCTAGATG			
4	DGR_D4	CGGAGTTTCTTTATCGTGATCC	205-273	P	0.6691
		ATACCAAGAGCTTCAGGCATTC			
5	DGR_D5	AGAAGCAGCAAACAACTCAACA	524-590	P	0.4198
		ACTGCATAGAGTGATGGGGATT			
6	DGR_D6	CTCATCACCTTTTCCAATTTCC	-	N/A	-
		GCAACCTTTCATTTTGTCCTGT			
7	DGR_D7	GGAGCAGCACTTCAATTCTTTT	239	M	-
		CATGGTGATTTTCATCTCTCTCTC			
8	DGR_D8	CCCTCTCTCTCTCTCTCGTG	239-274	P	0.4161
		ATCTGCTTTGGTTATGGGTTTG			
9	DGR_D9	ATTTTGGATCAGGCAACGTC	403-466	P	0.6401
		TTAATGTTTCAGCAAGTGACCC			
10	DGR_D10	GAAGGACTGGATGATTTGGAAC	223	M	-
		GGAAAATTAAGACGCACACACA			
11	DGR_D11	AGCTCCAAGCAATCAGAGAAAC	-	N/A	-
		GACTCCCTCCATAGGTTGAATG			
12	DGR_D12	TGATTGCGACACCAATAAACTC	-	N/A	-
		CACCATCACTACCTTCTCCTCC			
13	DGR_D13	TCTCTTTTCCCTTCTCTCTCCC	-	N/A	-
		ATCCTCTCTCTTTCTCTCCCGT			
14	DGR_D14	TCTCATCCTCTTCCTCTCTTCCT	-	N/A	-
		CCACTCCCCTTTCTTTCTCTCT			
15	DGR_D15	AGAGGCTTCAGAGTAGGGGAAT	92-364	P	0.7239
		GCAACATAGAGTAATCAACAAGGG			
16	DGR_D16	TTTTCTCTCTCTCCCCTCCTCT	165-182	P	0.3743
		CCCTCTTCTATCTCTTTTCCCC			
17	DGR_D17	CCGTCTTTCTTTCTCCCTCTTT	230	M	-
		TCCTTTCTCCTCTCTTTTCCCT			
18	DGR_D18	AGAGGAAGAGAGAGAAGGGAGG	440	M	-
		AAAACTCCTTGTTGGTCACTGG			
19	DGR_D19	GTAAAGTCACCAAGCATCCTCC	-	N/A	-
		TCCTTCTCTCTCATCTCCTTCTG			
20	DGR_D20	TCCTCTAAAACTCGGATTCTGC	-	N/A	-
		CCAAGGTAAGGGTAAGGGTACA			
21	DGR_D21	TATCGAGTTCAAAATCCACCG	-	N/A	-
		ACGGTTTCTCCCTCTCTTTTCT			
22	DGR_D22	TAAGCCATTCCTGCCCTATAAA	538-662	P	0.7024
		TGCCCTAAATCACCCTAACCTA			
23	DGR_D23	TTTGCTCAAGTCCCTCAATTTC	492-583	P	0.8195
		TGCCCTAAATCACCCTAACCTA			
24	DGR_D24	CACCAGATTGGACGAGTTCTCT	-	N/A	-
		GAGCAATTACCCAGATCAGTCC			
25	DGR_D25	TTGGGTCGCTATAAAGGTTTTG	422	M	-
		TGTTGTTGGTGCTTCCATAAAG			

Table 3 Continue ...

26	DGR_D26	TTAGACCATCACATGCTTCCAC	278	M	-
		GAGTTGTTGCTGCTGTTAGTGC			
27	DGR_D27	TTAGACCATCACATGCTTCCAC	358	M	-
		GAGTTGTTGCTGCTGTTAGTGC			
28	DGR_D28	TAATGCACCAAACAACCTCATC	-	N/A	-
		TACAGTGGCAAACGAGTTCATC			
29	DGR_D29	TCATCGGAACCTTGAAATGAC	336-398	P	0.3663
		GATTGTTGCTGCTGATTCTGTT			
30	DGR_D30	AAATAGCCGAACCTACCCTCTC	-	N/A	-
		GCTTTTGTCTTCCCTTTTCCTT			
31	DGR_D31	TAAGTTCAGGGCTCCTCTCATC	263-321	P	0.6553
		GTGTTTGAGCACATTGGAGGT			
32	DGR_D32	CCACAACAACAAAGACAACCAT	-	N/A	-
		TTTAATTGACACCTCAGCCTCC			
33	DGR_D33	GAGAATGCAAGAAACGAAAGGA	222-295	P	0.7706
		CGGTAAAATCCATCGGGATAGT			
34	DGR_D34	CCAGCATGTAACCATCAAAGAA	250	M	-
		ACTGACACTATCTGCAAGGCAA			
35	DGR_D35	AGCAAGCAAGGCAGAAGAGTAG	435	M	-
		GCGACTTCGAGATAGTGGTCTT			
36	DGR_D36	AAGACCACTATCTCGAAGTCGC	-	N/A	-
		CACAGAAACATCAAGCATTCGT			
37	DGR_D37	AGCAAGCAAGGCAGAAGAGTAG	190-502	P	0.5335
		GCGACTTCGAGATAGTGGTCTT			
38	DGR_D38	AAGACCACTATCTCGAAGTCGC	366	M	-
		CACAGAAACATCAAGCATTCGT			
39	DGR_D39	AGAGGAAGAGGAAGAAGAACGTG	145-203	P	0.6574
		CGGTAAAATCCATCGGGATAGT			
40	DGR_D40	GCACTGGTTAATTCATGTGTCAAG	309-388	P	0.5843
		CCAGAGGTTTGAGCCCTTTT			
41	DGR_D41	CATCTTCATCTTCTTCTGCACG	223	M	-
		CATGGGTGTTGTGTTATTTTGC			
42	DGR_D42	GGAGATATGGAGGTGGTTTGTC	243	M	-
		AATGGAGTCATCATCATTGTGC			
43	DGR_D43	CAAAATTACTCTCTGGATCACGG	326	M	-
		TATGAGGAGCCTTTAGGAGCAC			
44	DGR_D44	AACCGATCAATTCACATAACCC	228-365	P	0.5802
		CAAAGGGCGAAAAGTAGAAGAA			
45	DGR_D45	AGAAGCAGCAAACAACTCAACA	191-322	P	0.5040
		ACTGCATAGAGTGATGGGGATT			
46	DGR_D46	TCTCACAACTGCACCAGAAACT	269-337	P	0.3750
		ACACCAACAACTCCTCCTTCAT			
47	DGR_D47	GCTAGAGCTTACAAGGAACCCA	169-200	P	0.3750
		AAGTATGAAATGGATGGGGATG			
48	DGR_D48	AAAGGTGAGAAAAGGAGGAAGG	138	M	-
		ACGGTCGTCCCTAAAATTACAA			
49	DGR_D49	ATTAGAGGAGCGTCCAGAATCA	112-261	P	0.4604
		GACTTCTTGTCATAGGCAACCA			
50	DGR_D50	TTCCCTTATCCATTCATGCTCT	267-333	P	0.3812
		TATTTAGAAAACTTGCACGCCC			
51	DGR_D51	CAATTTCTGATGGGGCCTAA	131	M	-
		TGGGGTGAATCTGTTTTCTTTC			

Table 3 Continue ...

52	DGR_D52	TCCTTCTTCTCCTTCTCCTCCT	166	M	-
		TACTCACATGGCTGCTGTTCTT			
53	DGR_D53	AATGAGAATCACCGAATAACCG	-	N/A	-
		ACGAGGTACTGGAGGAGGCTA			
54	DGR_D54	ATTAACGGTGGGGTAACATTGA	-	N/A	-
		TCGAATTAGTAGGGGAAAACGA			
55	DGR_D55	TGAGTTGCAGAAGCAGAAGAAG	-	N/A	-
		ATTGAGGTGTTGGATGAGAGGT			
56	DGR_D56	TGAGTTGCAGAAGCAGAAGAAG	-	N/A	-
		GAGGAGGTAGAGTCGGAATTGA			
57	DGR_D57	GGAGGAGTAAGGGAGCAGGTAT	-	N/A	-
		ACCCTACAATCCATCATCCAAG			
58	DGR_D58	AGTCCTGGGGTTCTACACAAAA	-	N/A	-
		GAAGAGGGCTGAACAAGACACT			
59	DGR_D59	ATCTGCATCACTCCCAAGAACT	-	N/A	-
		CTTGTGTTCCTCGTGTCCTCTA			
60	DGR_D60	AACAAATTAGACCCAAGGATCG	306	M	-
		CTCTTCCTCTTCTTCCTCCTCA			
61	DGR_D61	GGTTCATCCTCCTCCAAATAA	127-234	P	0.2764
		CTCTTCCTCTTTCTTCTCTTCATC			
62	DGR_D62	ATTTTGGATCAGGCAACGTC	235-281	P	0.7445
		TCCTCCTCTTCTTCCTCCTTCT			
63	DGR_D63	GATGATGATGGAGGAGAAGGAG	215	M	-
		TTAATGTTTCAGCAAGTGACCC			
64	DGR_D64	TCTCCCATATCCACCATAGACG	274	M	-
		GGCCAGTCCTCAAGAGCTATC			
65	DGR_D65	CAAATCTTAATGGCTTCGGC	385	M	-
		GTTAGTGTCGAAAGTGAATTGTGC			
66	DGR_D66	GCAGAACTTATCATCACACATACATCG	246	M	-
		TGCAAAACTCTTCTTCTCCTTCCT			
67	DGR_D67	AGAATCGAACCACACCTCAGTC	208	M	-
		GCACGTTCTTCTTCTCTTTCGT			
68	DGR_D68	TGATTGCGACACCAATAAACTC	303-351	P	0.6382
		CACCATCACTACCTTCTCCTCC			
69	DGR_D69	GACGATGACGATAACAATGATG	-	N/A	-
		TTCTCTTTCCTTCTCCTTCTCC			
70	DGR_D70	TTAACATCCCTCCCTTCCCTAT	-	N/A	-
		TAGAAGTGGTCTTGATGGGCTT			
71	DGR_D71	CGAATACACACATCCATCCATC	-	N/A	-
		TCTTTCTTGAGGTTTCTCTGGC			
72	DGR_D72	GTGGTTGTTGTTGTTGGATTTG	194-249	P	0.4498
		CCAGTCCACTTCTTCTTCTTCC			
73	DGR_D73	TCTTACTCAGCTTCTGGGGTTG	254-280	P	0.0966
		GCAAAATAACAAGAGGGACGAA			
74	DGR_D74	TCTTCGTGTTATACCCATCTTCG	319-375	P	0.5681
		CTCCTTCTCATCTTCTGCTGCT			
75	DGR_D75	AGAGTTGTGGGTAGCGTGTTTT	226-251	P	0.3997
		CGAATAAGAGAGGAGAAATGCTAGA			
76	DGR_D76	CGAATAAGAGAGGAGAAATGCTAGA	259	M	-
		AGAGTTGTGGGTAGCGTGTTTT			
77	DGR_D77	ATTTTCACAGAGAGGATGGGAA	302-361	P	0.3749
		TGGGTCGTCCAAGTAATAAACC			

Table 3 Continue ...

78	DGR_D78	ATTGGAGGATGTGAATTGAACC	345	M	-
		ATCGCAATATGAGTGGCATGTA			
79	DGR_D79	TTCTCTTGAGCTTGAAAGGGAC	-	N/A	-
		ATAAAGCCCTCCATTCTTCCTC			
80	DGR_D80	GTAAAGTCACCAAGCATCCTCC	-	N/A	-
		TCCTTCTCTCTCATCTCCTTCTG			
81	DGR_D81	TCTTCCCCATAAACCTACCTCA	-	N/A	-
		GCTTTTGTCTTCCCTTTTCCTT			
82	DGR_D82	CTATACCTCATGTTCAGGCCCA	206	M	-
		GATCGCTTTGTCCTTCGTAAAA			
83	DGR_D83	AAAATGGACAAGGACAGGATTG	383	M	-
		CATAAAGTCGGATAGGATTGGG			
84	DGR_D84	GTTAGCACGGGTATTAAGCAGG	235	M	-
		ACCCTCACCACTACCAACACA			
85	DGR_D85	TCTCATTTCACCTCCTTCCATC	189	M	-
		CAAGAATGTCCTTATCCTCGCT			
86	DGR_D86	CACAGCTATTGTGTTTGTGGTG	390	M	-
		ATTCTGCCTCTCCTATCTTACAACT			
87	DGR_D87	ATCTCACATCTCTTCCATCCGT	352	M	-
		TGCAGTAAAATCACCAAGCATC			
88	DGR_D88	TTCACACAACACACCCCTCTAC	204	M	-
		CTTTTGTCTTCCCTTTCCCTCT			
89	DGR_D89	CTTCGGCCATCAGTAAGAAAGT	238	M	-
		CTGGAAAGACCTGGATGTTAGC			
90	DGR_D90	GTCACGTACCTATCGTACCCCA	389	M	-
		TGCACTATCCGCAAGGAAAATA			
91	DGR_D91	GGTACATAAACGGTCTCAAGCC	394	M	-
		TTTCTTGGTGGTGATAGCTGTG			
92	DGR_D92	ACTGACCCTGGCATTTGATAAG	325	M	-
		CCACGAGGATTGTTCGATTTAG			
93	DGR_D93	CAACGAGCAGAAGAGAAGAAAA	116	M	-
		ACTTAACCAAGTCATCCAAGCA			
94	DGR_D94	CTCCAACATTGCGATTTCCTTA	190	M	-
		AATTCAGACCCATCCTAGCCTT			
95	DGR_D95	TTCACCAACCAAGTCAGATACG	365	M	-
		TAAGAGGAGGACAACAACGACA			
96	DGR_D96	TTCTTCTTCAACCAAGTCGTCA	284	M	-
		ATGTGGTCATTTTGTGATGTGC			
97	DGR_D97	TTAAATTGGTCCCCTACGTTTG	89	M	-
		CAATGTCACCTCTCTAGTTGCG			
98	DGR_D98	TATCGAGTTCAAAATCCACCG	342	M	-
		ACGGTTTCTCCCTCTCTTTTCT			
99	DGR_D99	CTTTTGTCTTCCCTTTCCCTTT	246	M	-
		ACCTCATAAACCCCACCTACCT			
100	DGR_D100	CGGAGACAAGGGTGTAGATTG	187	M	-
		CCAACTCTGGTACAAAATCCAG			
101	DGR_D101	ACCTCATAAACCCCACCTACCT	357	M	-
		CTTTTGTCTTCCCTTTCCCTTT			
102	DGR_D102	ATGTTCTTATCCTTGCTCCTGC	127	M	-
		CTCATCTCACATCTCTGCCATC			
103	DGR_D103	AGTTTTGATGAGGTGCTTGGTT	257	M	-
		AAGATAGGAAAGATGCTCACGC			

Table 3 Continue ...

104	DGR_D104	GATTCGATTGGGAGACAAACTC	235	M	-
		TGAGAATTTTGGTGTTGACCTG			
105	DGR_D105	ACACACCCCTCTACACACTCCT	116	M	-
		AGGATTCTCCGGTCTTAGGTTC			
106	DGR_D106	TACCAGAGCAAGTGGACAACAC	257	M	-
		GGATGAAGTTTAATGGGTGGAA			
107	DGR_D107	AGATGATGACGACAAGGAGGTT	155	M	-
		TGTTGATGGTAAGGATGCTACG			
108	DGR_D108	ATACACCCAGACCCGAGAGAG	130	M	-
		GACGATTGTGGTGAAGCAATTA			
109	DGR_D109	ATCCCTTTATATGGTGGTGGC	397	M	-
		TCTATATCGTGGCATTGGATTG			
110	DGR_D110	CACCAGATTGGACGAGTTCTCT	-	N/A	-
		GAGCAATTACCCAGATCAGTCC			

M = Monomorphic, P= Polymorphic, N/A = No amplification, PIC = polymorphic information content

- Cluster III included TG-17, Tirupati-3, and TPG-41 (Fig. 2).

Genetic Diversity

The genetic diversity of the 96 groundnut genotypes was assessed through similarity index and cluster analysis using Free Tree software. The genotypes were grouped into three main clusters: Cluster I, Cluster II, and Cluster III, with an average similarity of 0.02.

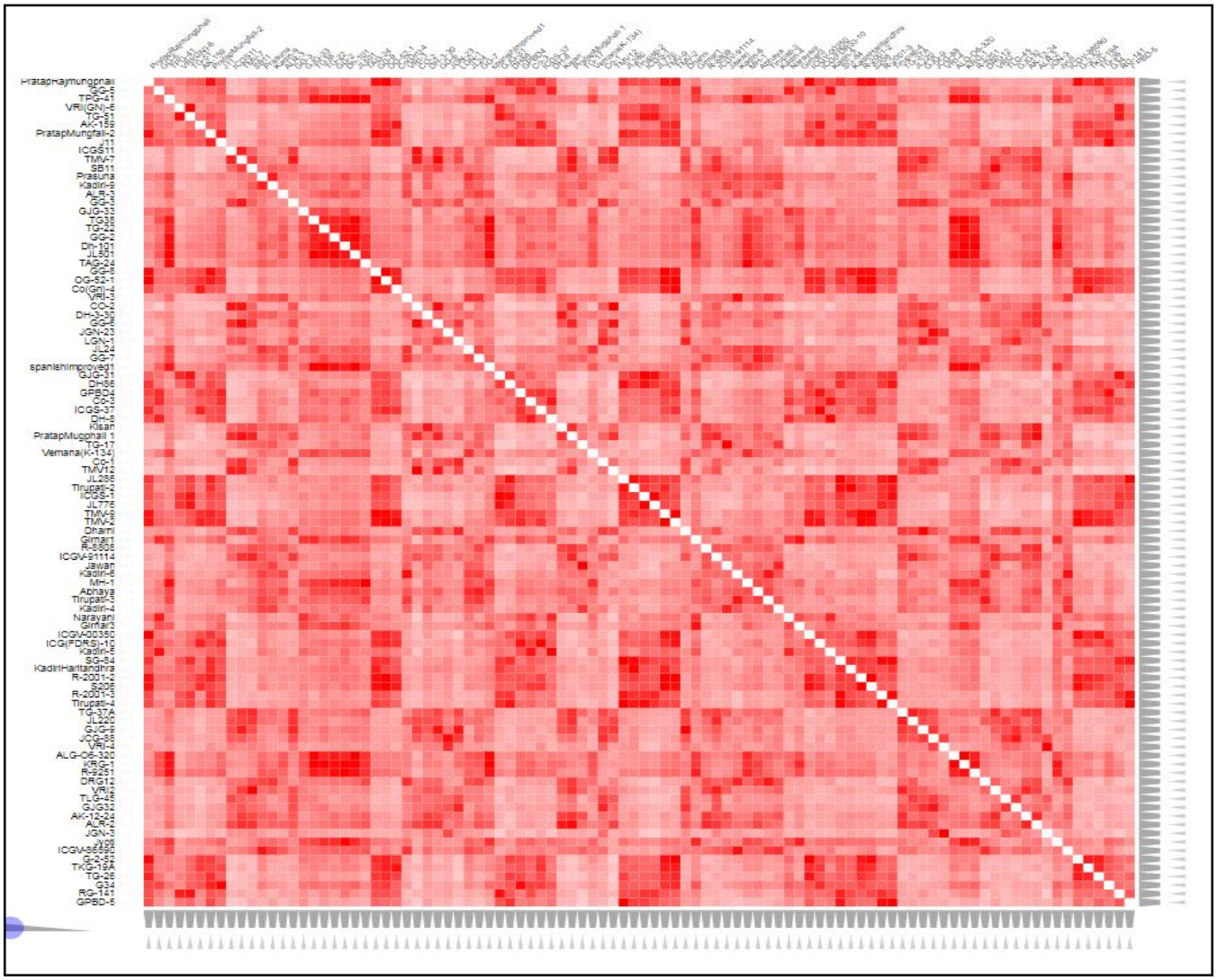

Fig. 1: Heatmap of Similarity matrix of 96 groundnut genotypes.

The similarity matrix analysis revealed that the highest similarity value was 0.9211 between the genotypes Spanish Improved and JL 501, while the lowest similarity value was 0.1552 between TMV-12 and DH86. The similarity matrix is represented as a heatmap in Fig. 1, showing the genetic relationships among the 96 groundnut genotypes.

Dendrogram and Cluster Analysis

The dendrogram based on the similarity matrix grouped the 96 groundnut genotypes into three major clusters:

- **Cluster I:** Consisted of 33 genotypes, subdivided into two subclusters (I and II). Subcluster I was further divided into subgroup A (which was split into A1 and A2) and subgroup B. Genotypes such as GJG-31, JL776, ICGS-1, RG-141, and Kadiri Haritandhra were part of this cluster.
- **Cluster II:** Included 27 genotypes, divided into two subclusters. Subcluster I had genotypes like JGN-2 and JCG-88, while Subcluster II featured groups with genotypes such as Kisan, DH-3-30, and ICGV-91114.
- **Cluster III:** Contained 36 genotypes, again divided into two subclusters. Genotypes such as Kadiri-4, TG-17, and Jawan were part of Subcluster I. Subcluster II included genotypes like Narayani, GG-5, and DH-86.

Each cluster was further divided into smaller groups, highlighting genetic diversity among the genotypes. For example, Cluster I contained Kadiri Haritandhra, Tirupati-4, and JL286, while Cluster III featured Girnar 3, Girnar 1, and TAG-24.

Population structure

A structure analysis of 96 Groundnut germplasm

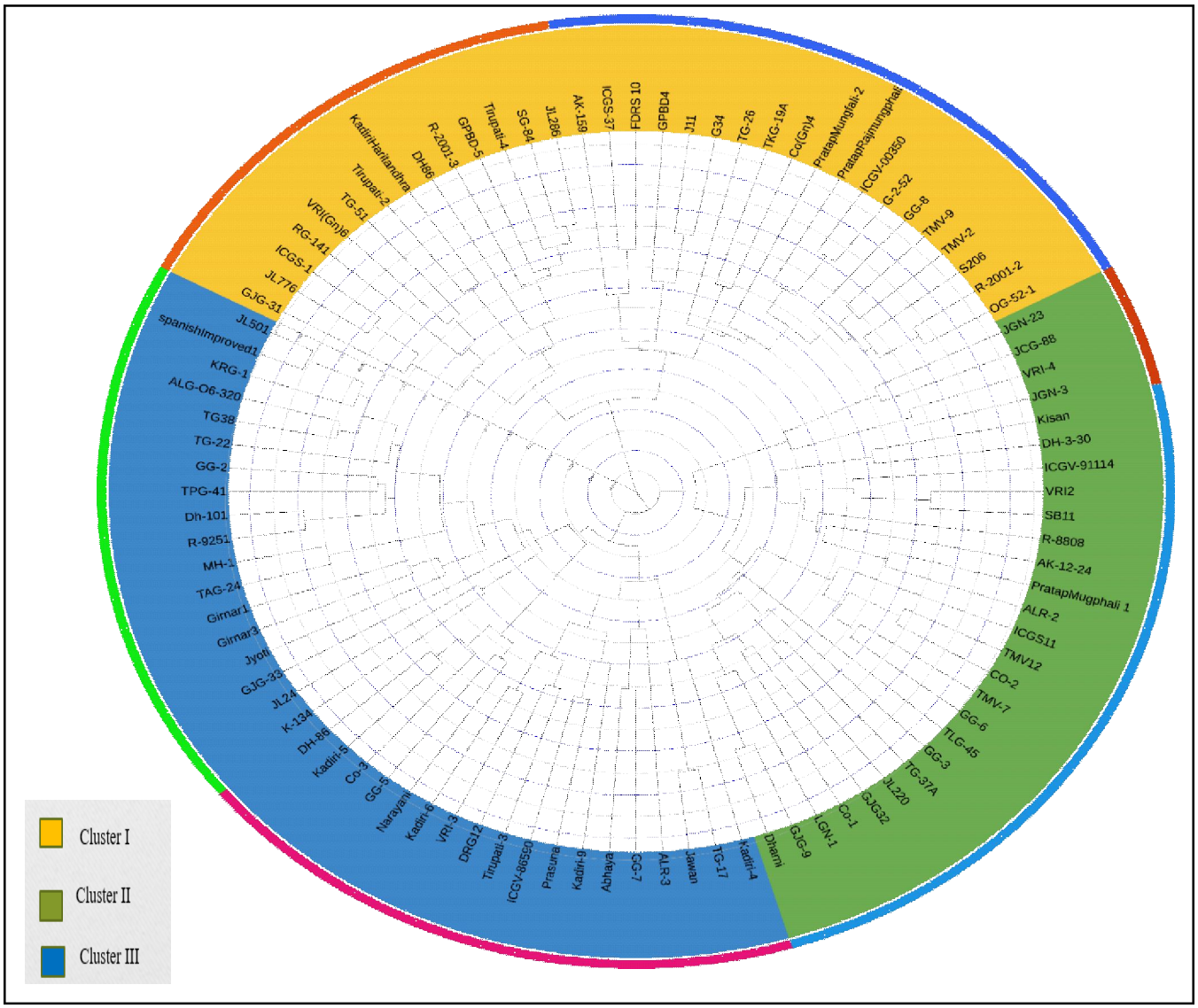

Fig. 2: Dendrogram of 96 genotypes based on similarity matrix

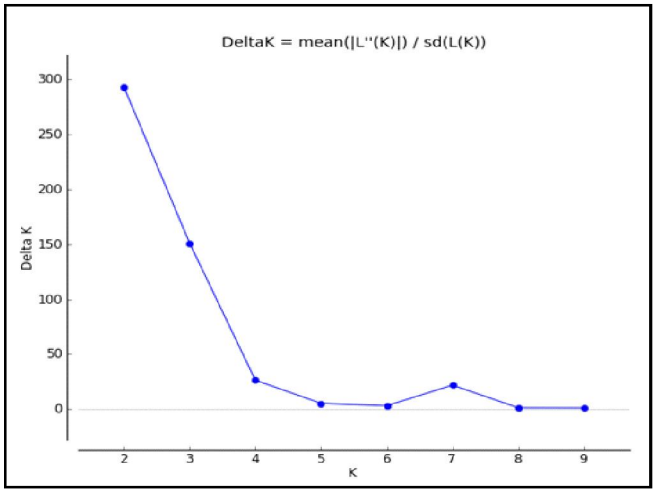

Fig. 3: Functional cluster and their magnitudes at *K=2* among 10 runs. **(X=k value); (Y= Number of runs)**

populations was conducted, varying K from 1 to 10. The likelihood values (Ln (PD)) decreased with increasing K, indicating clear population structure. The optimal number of populations was identified at K = 2, as shown in Fig. 3, using "Structure Harvester." A bar plot based on delta K revealed two subpopulations, represented in Fig. 4, which was further confirmed by a triangular plot (Fig. 5).

Accessions were categorized as pure (score > 0.80) or admixture (score < 0.80). In inferred population one (Cluster 1), there were 50 genotypes, while inferred population two (Cluster 2) had 46 genotypes. Details are listed in Table 4.

Clusters are denoted as 1 (red) and 2 (green), with segment lengths indicating genomic proportions. Average expected heterozygosity between individuals within clusters was:

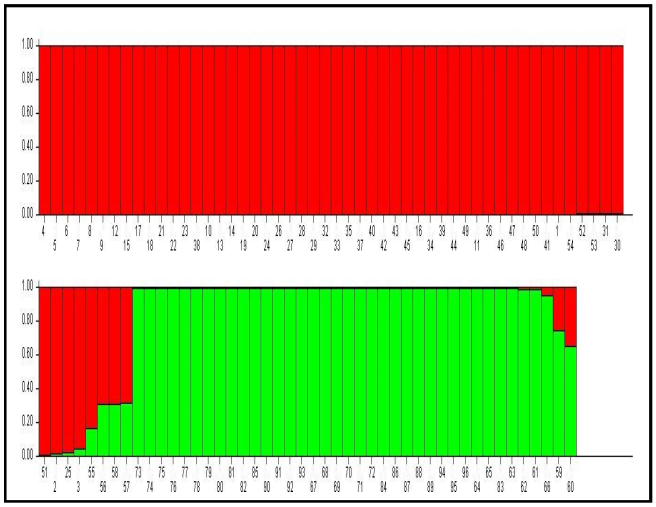

Fig. 4: Bar plot of the genetic composition of individual accessions of groundnut based on SSR marker generated by STRUCTURE 2.3.2 algorithm admixture model.

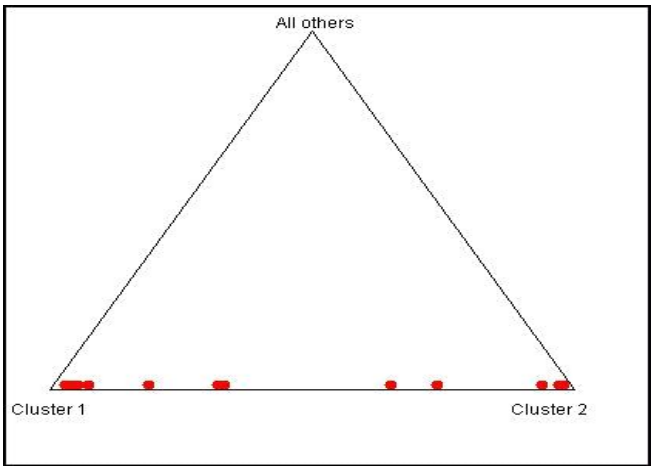

Fig. 5: Triangular plot of the genetic composition of individual accessions of groundnut based on SSR marker generated by STRUCTURE 2.3.2 algorithm admixture model.

Table 4: Inferred ancestry of individual.

S. No	Genotype	Cluster 1	Cluster 2
1	JGN3	0.994	0.006
2	VRI4	0.980	0.020
3	JGN23	0.952	0.048
4	JGN24	0.999	0.001
5	TLG45	0.999	0.001
6	GJG32	0.999	0.001
7	CO-1	0.999	0.001
8	LGN1	0.999	0.001
9	Dharni	0.999	0.001
10	GJG9	0.998	0.002
11	GJG6	0.996	0.004
12	TMV 7	0.999	0.001
13	TMV 12	0.998	0.002
14	CO2	0.998	0.001
15	ICGS11	0.999	0.001
16	GG3	0.997	0.003
17	JL220	0.999	0.001
18	TG37A	0.999	0.001
19	ICGV91114	0.999	0.002
20	VRI2	0.998	0.002
21	SB11	0.998	0.001
22	Pratap mughphali	0.999	0.001
23	ALR2	0.999	0.001
24	Kisan	0.998	0.002
25	DH330	0.975	0.025
26	AK1224	0.998	0.002
27	R8808	0.998	0.002
28	TG17	0.998	0.002
29	Jawan	0.998	0.002
30	Kadiri4	0.988	0.012
31	Kadiri9	0.990	0.010

Table 4 Continue ....

32	Prasuna	0.998	0.002
33	DRG12	0.998	0.002
34	ICGV86590	0.997	0.003
35	Tirupati	0.998	0.002
36	Kadiri6	0.996	0.004
37	VRI3	0.998	0.002
38	GG7	0.999	0.001
39	ALR3	0.997	0.003
40	Abhaya	0.998	0.002
41	JL24	0.995	0.005
42	Vemana (k-134)	0.998	0.002
43	MH-1	0.998	0.002
44	TAG24	0.997	0.003
45	TPG41	0.998	0.002
46	GG2	0.996	0.004
47	GL501	0.996	0.004
48	KRG1	0.996	0.004
49	ALGO 6320	0.997	0.003
50	TG38	0.996	0.004
51	Spanish improved	0.988	0.012
52	TG22	0.992	0.008
53	R9251	0.992	0.008
54	Dh101	0.993	0.007
55	Jyoti	0.831	0.169
56	Girnar 3	0.692	0.308
57	Girnar 1	0.682	0.318
58	GJG33	0.691	0.309
59	Narayani	0.254	0.746
60	GG5	0.348	0.652
61	Kadiri 5	0.010	0.990
62	DH8	0.008	0.992
63	CO3	0.004	0.996
64	GG11	0.003	0.997
65	G34	0.003	0.997
66	GPBD4	0.044	0.956
67	ICG (FDRS) 10	0.002	0.998
68	ICGS37	0.002	0.998
69	AK159	0.002	0.998
70	COGn4	0.002	0.998
71	TKG19A	0.002	0.998
72	TMV2	0.002	0.998
73	G252	0.001	0.998
74	ICGV00350	0.001	0.999
75	S206	0.001	0.999
76	R-2001-2	0.001	0.999
77	TMV9	0.001	0.999
78	OG521	0.001	0.999
79	TG26	0.001	0.999
80	Pratap Rajmugphali	0.001	0.999
81	GG8	0.001	0.999
82	Pratapmughphali	0.001	0.999
83	Kadiri haritandhra	0.003	0.997
84	Tirupati2	0.002	0.998
85	SG84	0.001	0.999
86	JL286	0.002	0.998
87	Tirupati4	0.002	0.998
88	GPBD5	0.002	0.998
89	R-2001-3	0.002	0.998
90	DH86	0.001	0.999
91	ICGS1	0.001	0.999
92	JL776	0.001	0.999
93	GJG31	0.001	0.999
94	RG141	0.002	0.998
95	TG51	0.002	0.998
96	VRI(GN)6	0.002	0.998

Table 4 Continue ....

Table 5: Analysis of molecular variance.

Source	DF	SS	MS	Est.var.	%
Among the population	8	146.129	18.266	0.149	2%
Among individuals	85	1296.903	15.258	5.469	55%
Within individuals	94	406.000	4.319	4.319	43%
Total	187	1849.032		9.938	100%

DF: degree of freedom, SS: Sum of square,
MS: Mean square, Est. var.: Estimation of variation

- Cluster 1: 0.5147
- Cluster 2: 0.4497.

Analysis of molecular variance

The total genetic variation in the 96 Groundnut genotypes was assessed, aligning with the population

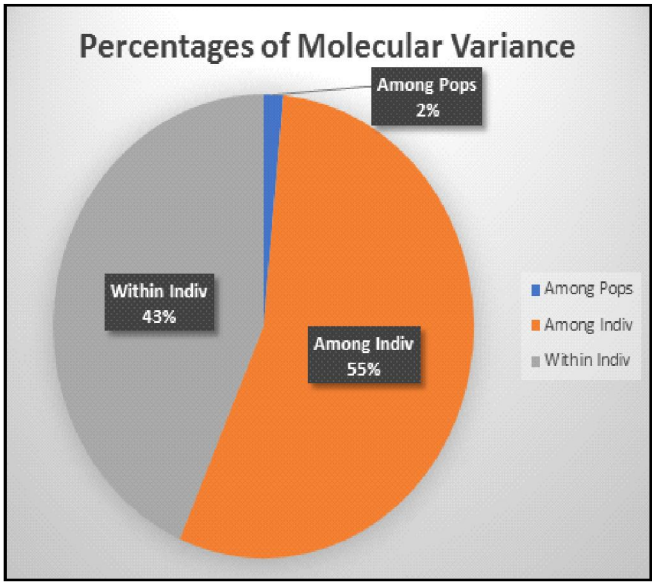

Fig. 6: Percentage of molecular variance in the Groundnut population.

structure analysis results. The AMOVA revealed that genetic variation among populations accounted for 2%, while variation among individuals was 55%. Molecular variance within the populations was 43%. A summary of the AMOVA is presented in Table 5 and Fig. 6**.**

Conclusion

This study highlights the successful development and validation of SSR markers to enhance groundnut (*Arachis hypogaea L.*) breeding programs, addressing critical challenges such as fresh seed dormancy, drought tolerance, and yield improvement. From 394 developed SSR markers, 110 were validated, with 30 exhibiting polymorphism and high utility for genetic diversity and population structure analyses. These markers have proven effective in identifying genotypes with desirable agronomic traits, offering a valuable resource for marker-assisted selection.

The analysis of genetic diversity and population structure revealed significant variation among the studied groundnut genotypes, emphasizing the genetic potential available for breeding programs. The clustering of genotypes based on functional markers provides a foundation for improving seed dormancy traits while enhancing tolerance to biotic and abiotic stresses. Importantly, genic SSR markers, due to their focus on functionally relevant genomic regions, demonstrated significant utility in breeding applications.

This research provides critical molecular tools to bridge the existing gaps in groundnut genetic improvement, particularly for traits associated with pre-harvest sprouting and environmental resilience. These findings contribute to the development of improved groundnut varieties, ensuring sustainable production and enhanced farmer livelihoods. Future research should expand the deployment of these markers in large-scale mapping populations and breeding programs to accelerate genetic gains in groundnut cultivation.

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