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Morphomolecular genetic study of selected groundnut genotypes with microsatellite marker

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Abstract

Morphomolecular genetic studies were undertaken to identify ten divergent genotypes of groundnut at molecular level using PM-36, PM50 and PM238 SSR primers. All the microsatellite loci amplified by Polymerase Chain Reaction (PCR) were found to be polymorphic in all groundnut genotypes except ICGV-99233. Differences were observed in the proportion of polymorphic loci, observed and expected heterozygosity values, average number of alleles in the cultivars. Only three genotypes showed significant deviations from Hardy Weinberg Equilibrium (HWE). The UPGMA dendrogram based on Nei's (1972) genetic distance, resulted in two major clusters with several sub clusters. Specific alleles of five varieties were identified (able to distinguish specific varieties), and these were PM-36/153 (Accession 12), PM-36/164 (BARI Badam 6), PM50/106 (Accession 12), PM50/102 (ICGV-93232), and PM238/100 (Accession 12). The three primers produced a total of 17 alleles with size ranging from 100bp to 164bp. The PIC (Polymorphism Information Content) value for the primer PM-36, PM50 and PM238 were found 0.75, 0.77 and 0.75, respectively. This approach will be useful for developing a set of number of SSR loci and for the protection of the varieties through Genetic Finger Printing (GFP).

Keywords: Groundnut, Microsatellite marker, Variety identification, *Arachis hypogaea*

Introduction

Cultivated groundnut (*Arachis hypogaea* L.) is an important crop for oil and protein source in Bangladesh. Nearly all *Arachis* species are diploid, but cultivated peanut (*Arachis hypogaea*) is an allotetraploid (genome AABB). It is a member of the section *Arachis*, which includes about 25 diploid and one tetraploid wild species (*Arachis monticola*) [Krapovickas and Gregorya 1994]. RFLP (Restriction Fragment Length Polymorphism), SSR (Simple Sequence Repeats) and different molecular analysis that included 17 diploid species of the section *Arachis* and three tetraploid *Arachis hypogaea* accessions suggested that a single origin for domesticated peanut and ancestral species related to *Arachis duranensis* (A genome) and *Arachis ipaensis* (B genome) as the most likely progenitors of *Arachis hypogaea.* [Kochert *et al.* 1996]. Cultivated peanut exhibits a little amount of variability for barriers to gene flow [Young *et al.* 1996], recent polyploidization from one or a few individual(s) of each diploid parental species or combined with self-pollination [Halward *et al.* 1991] and narrow genetic base [Singh and Singh 1992] These variations have been detected at the DNA level by using techniques of SSRs, RAPDs, AFLPs and RFLPs. For overall advantages, SSRs are important tools for identifying the little amount of variation in groundnut genotypes.

Molecular markers such as AFLP (Amplfied Fragment Length Polymorphism) and RAPD (Random Amplified Polymorphism DNA) have been used to evaluate germplasm bank accessions [He an Prakash, 1997, Gimenes *et al.* 2000, Dwivedi *et al.* 2001]. Data obtained with such markers has permitted the characterization and quantification of the genetic variability of germplasm but since these markers are dominant only one allele per locus can be detected, leading to an underestimation of the genetic variability. These types of markers have problems regarding repeatability and can result in the detection of many loci in a single assay, making it more difficult to assign allele to their loci [Powell *et al.* 1996].

The general objectives of this study were: (1) to identify suitable new SSR markers for genetic analysis of cultivated peanut (*Arachis hypogaea*), (2) to employ a set of SSR markers to analyze the genetic variation among some selected peanut genotypes, (3) to detect the locus position according to the number of close relatives and their phylogenetic position, (4) to evaluate the cross-species transferability of SSR markers and their usefulness in phylogenetic studies of the genus *Arachis.*

Materials and Methods

A total of ten different selected genotypes, four from ICRISAT (ICGV-99233, ICGV-01260, ICGS-E-55, ICGV-93232); two from BARI (BARI Badam 6 and Accession 12) and three from BINA (BINA Badam 1, 2, and 3) which were used as experimental material to study the genetic divergence and interrelationship among them by SSR markers.

Genomic DNA was extracted from the juvenile leaves (unfolded) of 30 days old plants according to the method described by Michiels *et al.* (2002) with some modifications [Extraction buffer (pH= 8.0): 50 mM Tris-HCl, 25 mM EDTA (Ethylenediaminetetraacetic acid), 300 mM NaCl and TEN buffer + 5% SDS (Sodium Dodecyl Sulfate) +10% PVP (Poly Vinyl Pyrolideone) +20% CTAB (Cetyl Trimethyl Ammonium Bromide)].The extracted DNA pellets were resuspended in 30-50 μl TE [10 mM Tris-HCl, and 1 mM EDTA], and quantified on 1% (w/v) agarose gels using known concentration of DNA as standards, based on which the extracted DNA was diluted to 25ng/μl and treated with 2 μl of RNAse for removing RNA.

PM 3, PM 36, PM 50, PM 210, PM 238 were first selected from the already available literature based on the polymorphic nature of the primers and after testing in the laboratory a set of three primers (PM 36, PM 50, PM 238) were finally selected (Table 1). DNA amplification were carried out in 25μl reaction mixtures, each containing genomic DNA (25ng/μl)=4 μl (100ng), Ampli Taq polymerase buffer (10X)=1 μl, Primer=2.5 μl (10 μM), dNTPs(250 μM)=1 μl, Ampli Taq DNA polymerase =1 unit (Banglore Genei, India) and sterile deionized water to prepare 10 μl reaction mix. The amplification conditions were originally recommended by Na-Nakorn *et al.* (1999) and Yue *et al.* (2003) with some modifications of annealing temperature and genomic DNA content. The annealing temperature of the three primer pairs were adjusted to 48ºC for PM-30, 56ºC for PM50 and PM238 [Guohao *et al.* (2003)]. The temperature profile consisted of 3 min initial denaturation at 94ºC followed by 35 cycles, each of 30 sec at 95ºC, 30 sec at the respective annealing temperature and 1min at 72ºC. Finally, an additional 5 min period for elongation at 72ºC followed the last cycle. The amplification products resolver on 6% polyacrylamide denaturing gels [25.24g urea (7M), 9 ml of 40% acrylamide: bis-acrylamide (19:1) and 12 ml 5 × TBE buffer were taken in a 100 ml beaker and deionized water was added to make the solution approximately 60 ml]^{PAGE} followed by silver staining. The stained photographic glass plates were air dried (2-3 days) then fragment sizes were calculated using white light. The computer programme SEQUAID II (Fragment sizer) by comparing with fragments of 100-bp ladder marker DNA.

Table 1. List of the screened primers

Locus	Forward primer	Reverse Primer	Ann. T.	Ref.
PM-3	gaa-aga-aat-tat-aca-ctc-caa-tta-tgc	cgg-cat-gac-agc-tct-atg-tt	55°C	Guohao *et al.* (2003)
PM-36	act-cgc-cat-agc-caa-caa-ac	cat-tcc-cac-aac-tcc-cac-at	50°C	Guohao *et al.* (2003)
PM50	caa-ttc-atg-ata-gta-ttt-tat-tgg-aca	ctt-tct-cct-ccc-caa-ttt-ga	50°C	Guohao *et al.* (2003)
PM 210	ccg-cag-at-ctt-ctc-ctg-tgt	cct-cct-cat-cct-cta-aac-tct-gc	55°C	Guohao *et al.* (2003)
PM238	ctc-tcc-tct-gct-ctg-cac-tg	aca-aga-aca-tgg-gga-tga-aga	55°C	Guohao *et al.* (2003)

Table 2. Deviation from Hardy-Weinberg expectations in 10 groundnut varieties (χ^2 values followed by degrees of freedom in parentheses[a])

Microsatellite loci	Varieties									
	ICGV-99233	ICGV-01260	ICGS-E-55	ICGV-93232	BARI Badam-6	Dhaka 1	BINA-Badam 1	BINA-Badam 2	BINA-Badam 3	Accesion-12
PM-36	ML	2.00NS 1	2.00NS 1	ML	2.00NS 1	2.00NS 1	2.00NS 1	2.00NS 1	2.00NS 1	2.00NS 1
PM-50	ML	5.33* 1	2.00NS 1	ML	2.00NS 1	5.33* 1	2.00NS 1	5.33* 1	2.00NS 1	2.00NS 1
PM-238	ML	2.00NS 1	2.00NS 1	ML	2.00NS 1	2.00NS 1	2.00NS 1	2.00NS 1	2.00NS 1	2.00NS 1

ML= Monomorphic locus: No further analysis
NS=not significant
*p<0.05

Allele frequencies were calculated directly from the observed genotypes and coded with a number ranging from 1 to n (number of alleles) (Table 3). Allelic variations and fit to Hardy-Weinberg proportions were estimated by the software POPGENE (version 1.31) by a chi-square (χ^2) test [Yeh *et al.* 1999] with 1000 simulated samples. The software DNA FRAG version 3.03 was used to estimate marker length and allelic length [Schaffer and Sedorf, 1981].Expected (H_e) and observed heterozygosity (H_o) were also calculated after [Nei, 1972] using the following formula and with the help of POPGENE (version 1.31) [Yeh *et al.* 1999] computer package program: $h_e = 2n\ (1- \Sigma\ x_i^2/N)/\ (2n - 1)$, where, h_e is the expected heterozygosity of each locus, n is the number of sampled individuals, x_i is the frequency of i-th allele at each locus and N is the number of loci examined. Thus, the average heterozygosity (H_e) was calculated as $He = \Sigma\ h_e / r$, Where, r is the number of loci examined (Nei and Roychoudhury, 1973). Nei's (1972) genetic distance value was computed using the formula as described in the POPGENE (Version 1.31) software user manual (Yeh *et al.* 1999).Genetic distance values (D) (Nei, 1972) were calculated as , $D= - \ln J_{XY}/\sqrt{\sum J_X J_Y}$, Where, $J_X=\sum X_i^2/ r$ in population X, $J_Y= \sum Y_i^2/ r$ in population Y, $J_{XY}=\sum X_i Y_i$, X_i and Y_i are the frequency of the i-th allele of a given locus in the two populations of fishes compared and r = the number of allele frequencies for all possible pairs of populations. The unweighted pair-group method with arithmetic mean (UPGMA) dendrogram was drawn by using the software TREEVIEW.

Results & Discussion

The number of alleles ranged from five to six per locus. The locus PM-50, and PM-36 had the highest number of alleles (six) while the locus PM-238 had the lowest number of alleles (five). The average number of alleles in ICGV-99233, Dhaka-1, and BINA-2 were the highest (Table 3). Guohao *et al.* (2003) found that the loci PM-36, PM-50, PM-238 were polymorphic having 7, 8 and 4 alleles per locus respectively in 24 genotypes of *Arachis.* They also studied polymorphic microsatellite loci in *Arachis hypogaea* collected from different location of Argentina, Bolivia, and Mexico and found allele per locus on an average 2-7 with mean heterozygosities 0.206-0.806. Primers pair allowed on an average the amplification in 63.33% (where from 0.00% to 100% in the Table 3) over the groundnut genotypes. In this study although some genotypes did not amplify certain loci, no relation was observed between failures to amplify, because different results were obtained with primers for loci from the same origin.

The data suggested that the loci amplified using heterologous primers had the same type of sequence as found in the total genotypes in respect to their levels of perfection and imperfection, because loci that had perfect sequences (sequences composed of uninterrupted repeats) in the total genotypes were more polymorphic than loci that had imperfect sequences (Table 3). The lower polymorphism in imperfect sequences has been suggested to be related to the lower probability of error during replication [Jones *et al.* 2001 and Hancock 2000] stated that reduced polymorphism in interrupted Sequences in compatible with the fact that incorrect pairing in that type of sequences is more difficult.

Total of the 10 Groundnut genotypes, 8 showed polymorphism (Table 3) and 2 were monomorphic. Significant deviations from Hardy-Weinberg Equilibrium (HWE) were detected in three out of 30 tests (Table 2). The deviation of PM-50 in V2, Dhaka-1, BINA-2 cultivar from Hardy-Weinberg Equilibrium was only significant.

The expected heterozygosity (H_e) ranged from 0.76 to 0.78 at the same genotypes (Table 4), and the observed heterozygosity (Ho) ranged from 0.10 to 1.00 .Microsatellite loci PM-238 showed highest Hardy-Weinberg average heterozygosity expected in subpopulation (H_t), and gene flow (N_m) estimated from F_{st} but lowest Hardy-Weinberg average heterozygosity obtained in subpopulation (H_s). Microsatellite loci PM-50 showed lowest Hardy-Weinberg average heterozygosity expected in subpopulation (H_t), and gene flow (N_m) estimated from F_{st} but highest Hardy-Weinberg average heterozygosity obtained in subpopulation(H_s), coefficient of population differentiation (F_{st}), observed & effective number of alleles. PM-36 only showed highest effective number of allele combine with PM-50. The observed heterozygosity (H_o) was low indicating that the species studied must be autogamous, or with very low cross fertilization rates. Valls *et al.* (1994) observed that most genotypes of the Arachis were autugamous.

Table 3. Allelic variation at the three microsatellite profiles of loci of PM-36, PM50, and PM238 in 10 groundnut genotypes

SL. No.	Cultivars	Band positions due to primers (bp)																			The No. of Polymorphic Locus over loci	The % of Polymorphic Locus Over loci
		PM 36							PM 50							PM 238						
		A	B	C	D	E	F	Genotype	A	B	C	D	E	F	Genotype	A	B	C	D	E	Genotype	
1	ICGV-99233						106	FF		121		112			BD	129					AA	0	0.00
2	ICGV-01260				140	111		DE	124						AA		118	112			BC	3	100
3	ICGS-E-55			148			106	CF			118				CC		118			104	BE	2	66.67
4	ICGV-93232					111		EE						102	FF	129		112			AC	1	33.33
5	BARI Badam-6	164					106	AF				112			DD	129		112			AC	2	66.67
6	Dhaka-1				140		106	DF	124						AA		118			104	BE	3	100
7	BINA-1				140		106	DF			118				CC	129	118				AB	2	66.67
8	BINA-2			148			106	CF			118				CC		118			104	BE	3	100
9	BINA-3			148			106	CF			118				CC		118			104	BE	2	66.67
10	Accesion-12		153			111		BE					106		EE		118		108		BD	2	66.67
Allele frequency		0.05	0.05	0.15	0.15	0.20	0.40		0.13	0.07	0.37	0.23	0.10	0.10		0.20	0.35	0.15	0.05	0.25			
Diversity Index (PIC=1-$\sum Xi^2$)		0.75							0.77							0.75							
Number of Alleles		6							6							5							
Allele Range (bp)		106-164							102-124							104-129							

Table 4. Summary of heterozygosity and genetic variation statistics for all loci

Locus	Obs_Hom	Obs_Het	Exp_Hom*	Exp_Het*	Nei**	Ave_Het	H_t	H_s	F_{st}	N_m	*na	*ne	*I
PM-36	0.20	0.80	0.24	0.76	0.75	0.40	0.80	0.76	0.47	0.29	6.00	4.00	1.56
PM50	1.00	0.00	0.22	0.78	0.77	0.13	0.00	0.78	0.83	0.05	6.00	4.33	1.62
PM238	0.10	0.90	0.24	0.76	0.75	0.45	0.90	0.76	0.40	0.38	5.00	4.00	1.47
Mean	0.43	0.57	0.23	0.77	0.76	0.33	0.57	0.77	0.57	0.19	5.67	4.11	1.55
Standard Deviation	0.49	0.49	0.01	0.01	0.01	0.17	0.49	0.01			0.58	0.19	0.07

* Expected homozygosity and heterozygosity were computed using Levene (1949)

** Nei's (1973) expected heterozygosity

H_t=Hardy-Weinberg average heterozygosity Expected in subpopulation

H_s=Hardy-Weinberg average heterozygosity Obtained in subpopulation

F_{st}=Co-efficient of population differentiation

N_m= Gene flow estimated from F_{st} or F_{cs}. e.g., $N_m=0.25(1-F_{st})/F_{st}$

* na = Observed number of alleles

* ne = Effective number of alleles

* I = Shannon's Information Index

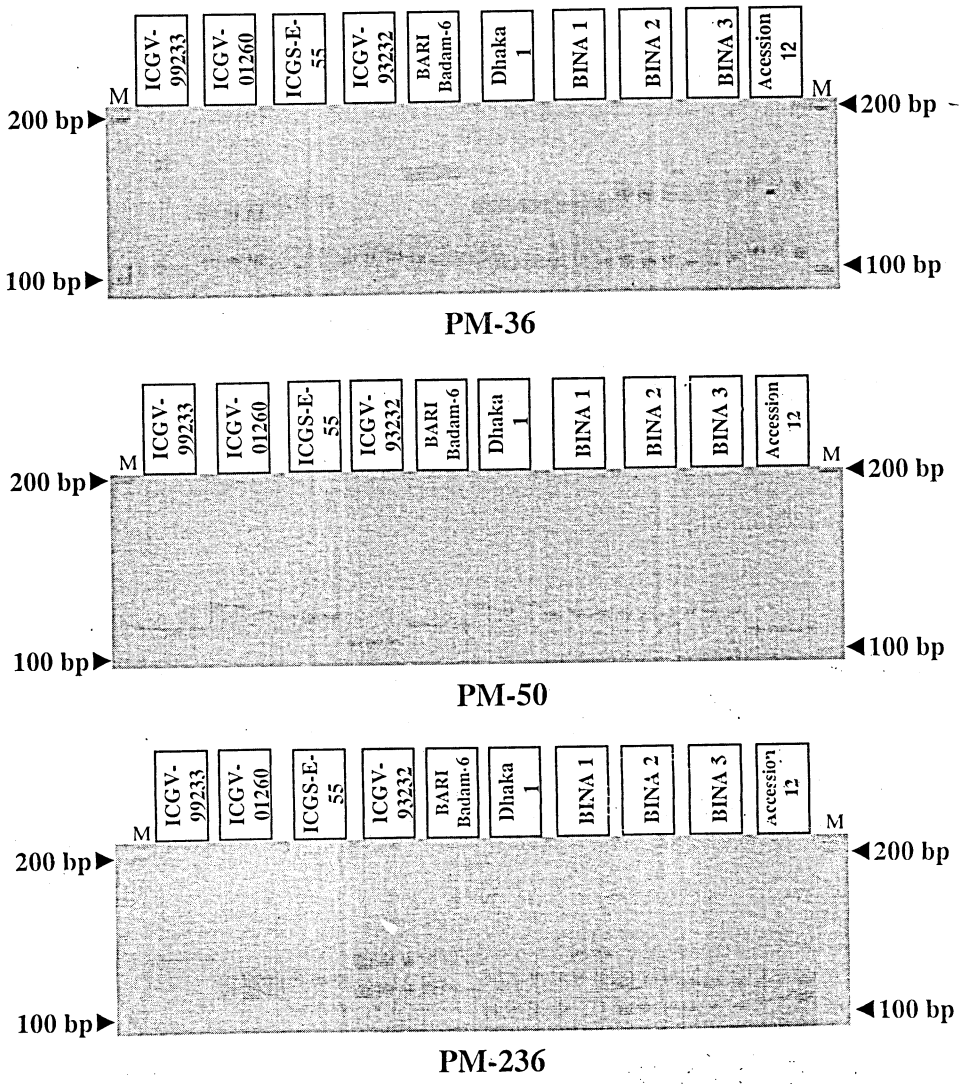

Fig 1. Microsatellite profiles of 10 groundnut (*Arachis hypogaea*) genotypes at loci, PM 36, PM 50, and PM 238. M: Molecular weight marker (100bp DNA ladder)

Highest Nei's genetic distance (2.0794) was estimated in ICGV-99233-BARI Badam 6;ICGS-E-55-BARI Badam 6; ICGS-E-55-Accession 12; BARI Badam 6-BINA Badam 1; BARI Badam 6-BINA Badam 3; BINA Badam 1- Accession 12; BINA Badam 3- Accession 12 cultivar pairs whereas, the lowest genetic distance (0.0000) was ICGV-99233-ICGV-01260; ICGV-99233-Accession 12; ICGS-E-55-ICGV-93232; ICGS-E-55-BINA Badam1; ICGV-93232-Dhaka 1;ICGS-E-55-BINA Badam 1; ICGS-E-55-BINA Badam 2; ICGS-E-55-BINA Badam 3; BARI Badam 6-Accession 12 (Table 5).

Table 5. Summary of Nei's (1972) genetic distance values between 10 groundnut cultivars

Cultivars	ICGV-99233	ICGV-01260	ICGS-E-55	ICGV-93232	BARI Badam-6	Dhaka-1	BINA-1	BINA-2	BINA-3	Accesion-12
ICGV-99233	****									
ICGV-01260	0.0000	****								
ICGS-E-55	1.5890	1.9538	****							
ICGV-93232	1.7006	0.9667	0.0000	****						
BARI Badam-6	2.0794	1.9538	2.0794	1.4979	****					
Dhaka-1	1.6346	0.3878	0.8552	0.0000	1.9538	****				
BINA-1	1.5890	1.2606	0.1335	0.0000	2.0794	0.5675	****			
BINA-2	0.9525	1.8281	0.0567	0.0000	1.1065	0 7295	0 2192	****		
BINA-3	1.5890	1.9538	0.0000	0.0000	2.0794	0.8552	0.1335	0.0567	****	
Accesion-12	0.0000	1.2606	2.0794	1.4979	0.0000	1.9538	2.0794	1.9538	2.0794	****

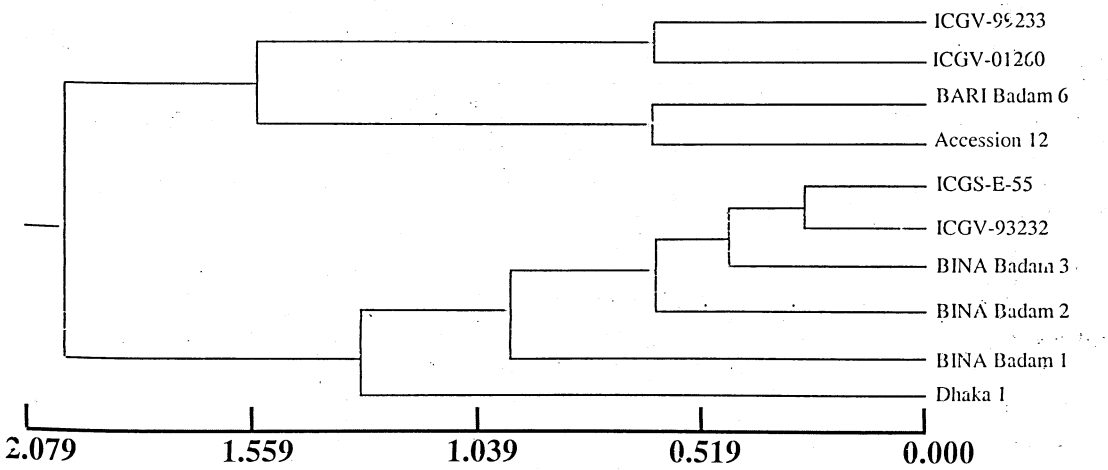

Fig. 2. UPGMA dendrogram based on Nei's (1972) genetic distance, summarizing the data on differentiation between 10 Groundnut genotypes according to microsatellite marker analysis

Geographical distance is an important factor that influences the genetic relatedness of populations [Wright, 1943]. UPGMA dendrogram based on Nei's (1972) genetic distance indicated segregation of 10 cultivars of groundnut into two main clusters: In cluster 1 ICGV-99233, ICGV-01260 in one grouped and BARI Badam 6, Accession 12 in another grouped with minimal genetic distance (0.000). Dhaka 1 grouped in sub cluster 1 of cluster 2 showed highest genetic distance values (2.023) with ICGV-99233. Subsequent separation of the second cluster occurred into sub clustering of BINA Badam 1, BINA Badam 2, ICGS-E-55 and ICGV-93232.

Conclusion

The results of the present study showed average number of alleles of all the groundnut genotypes were 1.67 over the three microsatellite loci. The coefficient of population differentiation (F_{st}) and gene flow (N_m) values across all the loci were 0.57 and 0.19 respectively. Significant deviations (χ^2 values followed by $P<0.05$) of PM-50 in ICGV-01260, Dhaka-1, BINA Badam-2 cultivar from Hardy-Weinberg Equilibrium (HWE) were detected. The Unweighted Pair Group Method of Arithmetic Mean (UPGMA) dendrogram based on Nei's (1972) genetic distance, resulted in two major clusters with several sub clusters. Through the present study five variety's specific alleles were identified (able to distinguish specific varieties) with sufficient level of genetic variation. These are, PM-36/153 (Accession 12), PM-36/164 (BARI Badam 6), PM50/106 (Accession 12), PM50/102 (ICGV-93232), and PM238/100 (Accession 12).The result of the present study would be useful to know genetic variation, population structure, parentage assessment, genome mapping, Marker Assisted Selection (MAS), forensics, stock purity, etc. of different populations of the studied species before undertaking any breeding program, and also will be used as baseline information for further study. However, more extensive molecular data are needed in order to draw conclusive remarks about the relationship between groundnut cultivars. Large number of samples would be necessary to determine if there are inherent differences in genetic distance between the groundnut cultivars. Moreover, using higher number of markers would give a clean idea about the genetic variation and genetic diversity which might be of greater interest for the plant breeders for the development of groundnut varieties.

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