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Genetic diversity analysis of groundnut genotypes using microsatellite markers

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Abstract

A set of groundnut microsatellite markers were utilized in 23 elite groundnut genotypes to assess the genetic diversity. A total of 13 alleles were detected at 3 loci using the PM3, PM50 and PM238 microsatellite primer pairs. The number of alleles ranged from 4 to 5 with an average of 4.33 alleles per locus. The allele sizes for all loci in 23 genotypes ranged from 137 to 217 bp and the frequency of SSR allele ranged from 0.022 to 0.500. The genetic distance for all possible 253 pairs of groundnut genotype combination ranged from 0.000 to 2.093 with an average of 0.92. The values of polymorphic information content (PIC) ranged from 0.617 to 0.701 and according to the result the primer PM3 was found to be the most polymorphic. The UPGMA dendrogram was constructed based on Nei's (1972) genetic distance delineated the above groundnut genotypes into two major clusters (I and II). Cluster I had two sub-clusters Ia and Ib and cluster II consisted of two genotypes namely, ICGV 94165 and ICGV 00340 were unique and diversed from all other genotypes belonging to cluster I. Regarding 3 primer pairs, 4 specific alleles (PM3/195, PM50/146, PM50/137 and PM238/171) were able to distinguish a maximum of 6 genotypes and finally 2 (ICGV 94165 and ICGV 00340) from the above 23 groundnut genotypes. This approach will be useful for exploiting SSR markers for detecting polymorphism leading to genotype identification and conservation of commercially developed groundnut varieties through DNA fingerprinting and for estimating genetic diversity.

Keywords: Genetic Diversity, Groundnut, Microsatellite DNA Markers

Introduction

Groundnut (*Arachis hypogaea* L.), also known as peanut, is one of the most important oil-yielding crops grown in 96 countries around the world including many tropical and sub-tropical countries (Krishna *et al.* 2004). Cultivated groundnut consists of two subspecies, *hypogaea* and *fastigiata*. Nearly all *Arachis* species are diploid (2n=2x=20), but cultivated peanut is an allotetraploid (2n=4x=40) with an AABB genomic constitution and aneuploid (2n=2x=18) species also have been described (Nimmakayala *et al.*, 2007). Molecular markers have been proven to be very powerful tool in the assessment of genetic variation for crop improvement. In peanut, the use of molecular markers such as RAPDs, AFLPs and RFLPs for breeding purposes has been limited for the low level of genetic variation has been detected at the DNA level (Subramanian *et al.*, 2000). Microsatellite or Simple sequence repeat (SSR) markers are the most commonly used markers because they are genetically defined, highly variable, co-dominant markers because of their ease of detection by PCR amplification of small amount of DNA (Moretzsohn *et al.*, 2004). These markers has been reported to be applied widely to detect genetic diversity, cultivar identification in different crop plants like Rice (Chakravarthi and Naravaneni, 2006), Wheat (Ahmad, 2006), Soybean cultivars (Islam *et al.*, 2007), Pigeon pea (Odeny *et al.*, 2007) etc. Recently, some studies revealed DNA polymorphism in *A. hypogaea* using SSRs to distinguishing closely related genotypes (Kameswara *et al.*, 2007; Krishna *et al.*, 2004; Guohao *et al.*, 2003). The application of microsatellite SSR markers has been found to be useful in characterization of released varieties of important crops of Bangladesh including 10 groundnut varieties (Rahman *et al.*, 2007). The objectives of the present work are to detect DNA polymorphism and identification of the groundnut genotypes, to estimate the relationship among groundnut genotypes and to utilize a set of three SSR markers to analyze the genetic diversity among 23 groundnut genotypes.

Material and Methods

The experiment was carried out in the Genetic Fingerprinting Laboratory, Department of Genetics and Plant Breeding and Central Laboratory of Bangladesh Agricultural University, Mymensingh, Bangladesh. A total of 23 drought tolerant groundnut genotypes collected from ICRISAT, India were sown in plastic pots for raising seedlings. Juvenile unfolded leaves were then collected from 25-30 day old plants taking three samples per variety in order to extract genomic DNA according to the method described by Michiels *et al.* (2002) with some modifications until DNA pellet precipitate. Finally, the DNA pellets were re-suspended in 30-35 μl TE buffer and treated with 2 μl of RNAse for removing of RNA. At last, the tubes with DNA samples were preserved at -20ºC. Then, the presence of genomic DNA was confirmed on 1% agarose gel and DNA concentration of each sample was quantified using spectrophotometer at 260 nm wave length.

A set of five microsatellite loci (PM3, PM36, PM50, PM210 and PM238) have been selected from the literature cited by Guohao *et al.*, (2003) to estimate the potentials of these markers for variety identification. Finally, 3 primers such as PM3, PM50, and PM238 were selected based on their performance in PCR amplification for SSR analysis. PCR was performed in a 10 µl reaction volume containing 50 ηg template DNA, 2.5 μM of each primer, 0.25 mM each of the dNTPs, 1 unit of *Taq* DNA polymerase, 1 µl 10 × reaction buffers and sterile deionized water. Amplification was carried out in an oil free thermal cycler (Thermal cycler gradient, Eppendorf). The annealing temperature of the three primer pairs were adjusted to 55ºC for PM3 and PM238; 50ºC for PM50. The temperature profile consisted of 3 minutes initial denaturation at 94ºC followed by 35 cycles, each of 30 sec at 95ºC for denaturation, 30 sec at the respective annealing temperature and 1 minute at 72ºC for extension. Finally, an additional 5 minutes period for elongation at 72ºC followed the last cycle. When the PCR was completed, the amplified products were kept in a refrigerator at 4ºC. PCR amplification was confirmed on 2% agarose gel. PCR products were separated on 6% denatured polyacrylamide gel containing 19:1 Acrylamide:Bis acrylamide and 7M urea. Electrophoresis was carried out on Sequi Gen GT eletrophoresis system (Bio-Rad, USA). SSR profile was visualized following progema silver staining protocol.

The bands representing alleles of a particular locus were scored manually and a single genotypic data matrix was constructed for all loci. Allele frequencies, allele numbers, estimation of Nei's genetic distance values (*D*), effective number of alleles and Shannon's Information Index were done by the software POPGENE (version 1.31) (Yeh *et al.*, 1999) with 1000 simulated samples. Polymorphism Information Content (PIC) was computed by adding the square values for all the frequencies of different alleles produced by a single marker locus and then deducted from one i.e., PIC = 1- ΣXi^2, where, Xi is the frequency of the *i*-th allele of a particular locus (Botstein *et al.*, 1980). The UPGMA dendrogram was drawn by using the software TREEVIEW based on Nei's, 1972 genetic distance. The software DNA FRAG version 3.03 was used to estimate allelic length (Nash, 1991).

Results and Discussion

In this study 23 genotypes of peanut were analyzed using 3 primer pairs (PM3, PM50 and PM38). Microsatellite profile of each locus is shown in Fig. 1. The locus PM50 had the highest number of alleles (5) while the locus PM3 and PM238 had the lowest number of alleles (4). The average number of alleles in ICGV 99231 was the highest (2) while that in other genotypes were 1.667 (Table 1). Guohao *et al.* (2003) tested groundnut genotypes such as *Arachis hypogaea, A. hirsute, A. fastigiata, A. peruviana, A. aequatoriana,* and *A. vulgaris* collected from 8 different countries using 19 microsatellite markers and also found that the loci PM3, PM50 and PM238 were polymorphic having 7, 8 and 4 alleles per locus, respectively among 24 genotypes of *Arachis.* The average number of alleles per locus was 4.25 and up to 14 alleles were found at one locus which is more or less similar to the present

study. Krishna *et al.* (2004) observed that the loci PM3, PM50 were polymorphic having 9, 7 alleles per locus, respectively and also studied 48 Valencia peanut genotypes collected from 20 diverse origin using 18 fluorescently labeled SSR (f-SSR) primer pairs. In the present study 3 primer pairs produced a total of 13 alleles. Rahman *et al.* (2007) also found six to seven alleles with mean 6.33 per locus by using 3 SSR loci (PM36, PM50 and PM250) in 10 groundnut varieties. Our study yielded comparatively less number of alleles than that of reported by Guohao *et al.* (2003), Krishna *et al.* (2004) and Rahman *et al.* (2007) because the collected groundnut genotypes are of same origin with narrow genetic diversity.

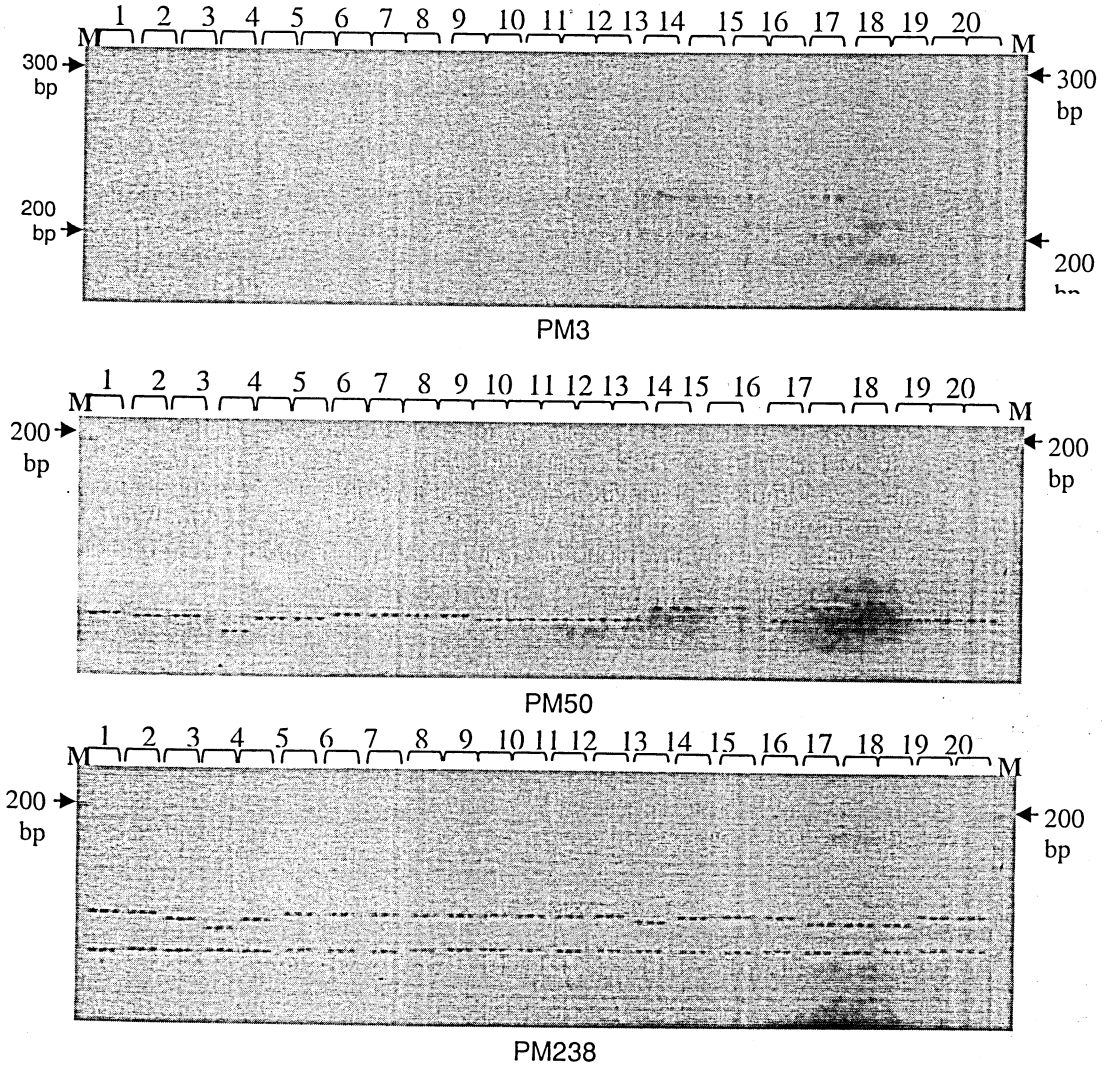

Fig. 1. Microsatellite profiles of 23 groundnut genotypes at loci, PM3, PM50 and PM238; The numerical numbers used for each locus indicate the groundnut genotypes used in the study purpose in the following way, 1: ICGV 94136; 2: ICGV 94138; 3: ICGV 94143; 4: ICGV 94165; 5: ICGV 94173; 6: ICGV 95399; 7: ICGV 95401; 8: ICGV 95412; 9: ICGV 95414;10: ICGV 96316; 11: ICGV 96318; 12: ICGV 97228; 13: ICGV 97229; 14: ICGV 97232; 15: ICGV 87846; 16: ICGV 99229; 17: ICGV 99231; 18: ICGV 99233; 19: ICGV 99235; 20: ICGV 00340; 21: ICGV 00343; 22: ICGV 00349; 23: ICGV 00350. Lane M: Molecular weight marker (100 bp DNA ladder).

Table 1. Size and frequency of alleles at three microsatellite loci in 23 groundnut genotypes

SSR loci	Allele size (bp)	Genotypes with allele frequency of an individual band pattern																						Average allele frequency	Diversity index, PIC=1-$\sum X_i^2$
		ICGV 94136	ICGV 94138	ICGV 94143	ICGV 94165	ICGV 94173	ICGV 95399	ICGV 95401	ICGV 95412	ICGV 95414	ICGV 96316	ICGV 96318	ICGV 97228	ICGV 97229	ICGV 97232	ICGV 87846	ICGV 99229	ICGV 99231	ICGV 99233	ICGV 99235	ICGV 00340	ICGV 00343	ICGV 00349	ICGV 00350		
PM3	217	0.5	0.5			0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.33		0.5			0.5	0.5	0.384	0.701
	207			0.5	0.67					0.5					0.5			0.5	0.5		0.5	0.5			0.181	
	202	0.5	0.5			0.5	0.5	0.5	0.5		0.5	0.5	0.5	0.5		0.5	0.5			0.5			0.5	0.5	0.326	
	195			0.5*	0.33*													0.167*	0.5*		0.5*	0.5*			0.109	
PM50	146																				1*				0.044	0. 673
	144																1	1		1					0.130	
	142	1						1	1	1	1								1			1	1	1	0.391	
	139		1	1		1	1					1	1	1	1	1									0.391	
	137				1*																				0.044	
PM238	175	0.5	0.5				0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5		0.5	0.5	0.5				0.5	0.5	0.333	0.617
	173			0.5		0.5										0.5				0.5	0.5	0.5			0.145	
	171				0.5*																				0.022	
	166	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.500	

* Specific allele of a locus for distinguishing specific genotype

The size of alleles for all microsatellite loci in 23 genotypes ranged from 137 to 217 bp (i.e. the loci PM3, PM50, PM238 had the base pair ranged 195-217 bp, 137-146 bp, 166-175 bp, respectively) as shown in Table 1. In PM3 locus, 217 bp alleles were present in 18 genotypes; 207 bp alleles in 8 genotypes; 202 bp alleles in 15 genotypes; 195 bp alleles in 6 genotypes. In PM50 locus, 146 bp alleles were present in only one genotype (ICGV 00340); 144 bp alleles in 3 genotypes; 142 bp alleles in 9 genotypes; 139 bp alleles in 9 genotypes as well as 137 bp alleles were only present in one genotype (ICGV 94165). In PM238 locus, 175 bp alleles were present in 16 genotypes; 173 bp alleles in 6 genotypes; 171 bp alleles in only one genotypes (ICGV 94165); and 166 bp alleles in 23 genotypes are shown in Table 1. The experiment showed that the genotypes ICGV 00340 and ICGV 94165 were different at 146 bp and 137 bp level, respectively from others in PM50 locus. Similarly, the genotype ICGV 94165 was dissimilar among them at 171 bp level in PM238 locus. Guohao *et al.* (2003) found that size of alleles for three loci (PM3, PM50 and PM238) in 24 peanut genotypes ranged from 94 to 224 bp i.e., the loci PM3, PM50 and PM238 had the base pair range from 200-222 bp, 94-224 bp and 164-170 bp, respectively. Krishna *et al.* (2004) observed that the size of alleles for two loci (PM3 and PM50) ranged from 99 to 221bp (i.e., the loci PM3 and PM50 had the base pair range from 188-221 bp and 99-114 bp, respectively) among 48 groundnut genotypes. Rahman *et al.* (2007) reported that the allele sizes of the three loci ranges from 109 to 241 bp in their study. The number and size of allele of PM3, PM50 and PM238 loci in peanut of various experiments in different places were observed variable due to geographical location, different species, reproductive isolation and analytical variation. The observed number of alleles in PM3, PM50 and PM238 were 4, 5 and 4, respectively and their mean was 4.33 and standard deviation was 0.58. The effective number of alleles in PM3, PM50 and PM238 were 3.35, 2.61 and 3.06, respectively with the mean of 3.01 and standard deviation 0.37. Shannon's Information Index in PM3, PM50 and PM238 were 1.28, 1.08 and 1.27, respectively with the mean of 1.21 and standard deviation 0.12 (Table 2).

Table 2. Summary of genetic variation statistics for all loci

Locus	*na	*ne	*I
PM3	4.00	3.35	1.28
PM50	5.00	2.61	1.08
PM238	4.00	3.06	1.27
Total	13.00	9.02	3.63
Mean	4.33	3.01	1.21
St Dev	0.58	0.37	0.12

* na = Observed number of alleles; * ne = Effective number of alleles; * I = Shannon's Information Index

The genetic distance among 23 groundnut genotypes were found from 0.000 to 2.093 (Table 3). The highest genetic distance (2.093) was found among ICGV 94165 to 14 pair genotypes. The lowest genetic distance (0.000) was found among ICGV 94136 to 5 genotypes; ICGV 94138 to 3 genotypes; ICGV 94173 to ICGV 87846; ICGV 95401 to 4 genotypes; ICGV 95412 to 3 genotypes; ICGV 96316 to 2 genotypes; ICGV 96318 to 2 genotypes; ICGV 97228 to ICGV 97229 and ICGV 00349 to ICGV 00350. The genetic distance (0.000) among 23 groundnut genotypes were inbred and the genetic distance (2.093) of the genotypes were relatively diverse. In the present study, four specific alleles (PM3/195, PM50/146, PM50/137 and PM238/171) were identified (able to distinguish specific genotypes) six genotypes out of 23 by three primer pairs. Among six groundnut genotypes, four (ICGV 94143, ICGV 99231, ICGV 99233, and ICGV 00343) were with PM3/195; one (ICGV 94165) was with PM3/195, PM50/137 and PM238/171 and one (ICGV 00340) was with PM3/195 and PM50/146. This study found that the genotype ICGV 94165 was identified with the three primers PM3, PM50, PM238 at the band level 195bp, 137bp and 171bp, respectively and the genotype ICGV 00340 was identified with the two primers PM3 and PM50 at band level 195bp and 146bp, respectively (Table 1), suggesting that these two genotypes were more specific among others.

Table 3. Summary of Nei's (1972) genetic distance values between 23 groundnut genotypes for all loci

Genotypes	ICGV 94136	ICGV 94138	ICGV 94143	ICGV 94165	ICGV 94173	ICGV 95399	ICGV 95401	ICGV 95412	ICGV 95414	ICGV 96316	ICGV 96318	ICGV 97228	ICGV 97229	ICGV 97232	ICGV 87846	ICGV 99229	ICGV 99231	ICGV 99233	ICGV 99235	ICGV 00340	ICGV 00343	ICGV 00349
ICGV 94138	0.693																					
ICGV 94143	2.079	0.470																				
ICGV 94165	2.093	2.093	0.995																			
ICGV 94173	0.981	0.134	0.288	2.093																		
ICGV 95399	0.847	0.059	0.316	2.065	0.014																	
ICGV 95401	0.000	0.693	2.079	2.093	0.981	0.847																
ICGV 95412	0.000	0.693	2.079	2.093	0.981	0.847	0.000															
ICGV 95414	0.134	0.981	1.386	1.246	1.386	1.204	0.134	0.134														
ICGV 96316	0.000	0.693	2.079	2.093	0.981	0.847	0.000	0.000	0.134													
ICGV 96318	0.693	0.000	0.470	2.093	0.134	0.058	0.693	0.693	0.981	0.693												
ICGV 97228	0.693	0.000	0.470	2.093	0.134	0.058	0.693	0.693	0.981	0.693	0.000											
ICGV 97229	0.693	0.000	0.470	2.093	0.134	0.058	0.693	0.693	0.981	0.693	0.000	0.000										
ICGV 97232	0.981	0.134	0.288	1.246	0.288	0.205	0.981	0.981	0.693	0.981	0.134	0.134	0.134									
ICGV 87846	0.981	0.134	0.288	2.093	0.000	0.014	0.981	0.981	1.386	0.981	0.134	0.134	0.134	0.2877								
ICGV 99229	0.693	0.693	2.079	2.093	0.981	0.847	0.693	0.693	0.981	0.693	0.693	0.693	0.693	0.9808	0.981							
ICGV 99231	1.070	1.070	1.204	1.126	1.540	1.329	1.070	1.070	0.752	1.070	1.070	1.070	1.070	0.7516	1.540	0.154						
ICGV 99233	0.288	1.386	0.981	0.995	2.079	1.763	0.288	0.288	0.134	0.288	1.386	1.386	1.386	0.9808	2.079	1.386	0.847					
ICGV 99235	0.981	0.981	1.386	2.093	0.693	0.752	0.981	0.981	1.387	0.981	0.981	0.981	0.981	1.3863	0.693	0.134	0.316	2.079				
ICGV 00340	2.079	2.079	0.693	0.995	1.386	1.540	2.079	2.079	1.386	2.079	2.079	2.079	2.079	1.3863	1.386	2.079	1.204	0.981	1.386			
ICGV 00343	0.470	2.079	0.693	0.995	1.386	1.540	0.470	0.470	0.288	0.470	2.079	2.079	2.079	1.3863	1.386	2.079	1.204	0.134	1.386	0.693		
ICGV 00349	0.000	0.693	2.079	2.093	0.981	0.847	0.000	0.000	0.134	0.000	0.693	0.693	0.693	0.9808	0.981	0.691	1.070	0.288	0.981	2.079	0.470	
ICGV 00350	0.000	0.693	2.079	2.093	0.981	0.847	0.000	0.000	0.134	0.000	0.693	0.693	0.693	0.980	0.981	0.693	1.070	0.288	0.981	2.079	0.470	0.000

The diversity index or polymorphic information index (PIC) were estimated and shown in Table 1. The values of PIC of the three loci ranged from 0.617 to 0.701, with mean 0.664 and the highest value of 0.701 was recorded for PM3 which was found to be the most polymorphic. A higher number of alleles per locus and the higher values of PIC found in the present study may be attributed to the use of more informative markers with relatively more diverse genotypes. Similarly, the low values of PIC and Marker index (MI) obtained in other earlier studies on wheat may be due to a smaller population (Roder *et al.*, 1995). The diversity index was found ranging from 0.76 to 0.815 by exploiting three loci with an average 0.79 while the locus PM238 was the maximum value which seems to be most polymorphic (Rahman *et al.*, 2007). In the present study, the estimates of genetic distance among 253 pairs of groundnut genotypes ranged from 0.000 to 2.093 with a mean of 0.92 (Table 3). The similarity matrix using Jaccard similarity coefficient revealed the highest similarity coefficient (0.91) between groundnut genotypes of Bulgarian origin while the lowest similarity coefficient of 0.16 was detected between PI 497288 of Bolivia and S-3663 of Bulgaria, the average similarity among accessions was 0.45 (Krishna *et al.*, 2004). The highest genetic similarities and low genetic distances among genotypes were suggested that they were closely related. This is why, in the present study the highest genetic distance value of 2.093 observed among groundnut genotypes (ICGV 94165 to ICGV 94136, ICGV 94138, ICGV 94173, ICGV 95401, ICGV 95412, ICGV 96316, ICGV 96318, ICGV 97228, ICGV 97229, ICGV 87846, ICGV 99229, ICGV 99235, ICGV 00349 and ICGV 00350) which suggested that they were highly diverse due to they belong to different ecotype or races, sub-species, semi-species and species.

The UPGMA (unweighted pair-group method with arithmetic average) dendrogram was prepared based on Nei's (1972) genetic distance delineated the above 23 groundnut genotypes into two main clusters as shown in Fig. 2 suggesting the existence of high level of diversity. Cluster I with 21 genotypes and cluster II with 2 genotypes. Cluster II containing unique genotype ICGV 94165 and ICGV 00340. According to their genetic distance, the remaining 21 genotypes belonging to cluster I again clustered into two sub-clusters; sub-cluster Ia and Ib containing 9 and 12 genotypes, respectively. Cluster II consists of ICGV 94165 and ICGV 00340 genotypes so that this two genotypes were found unique and diverse in origin with respect to all other genotypes belonging to cluster I. Fourteen groundnut genotypes showed highest genetic distance (2.093) with the genotype ICGV 94165. The dendrogram showed that the genotypes were closely related belonging to the same cluster while the genotypes ICGV 94165 and ICGV 00340 belonging to different cluster suggesting that these two genotypes were genetically diverse in origin.

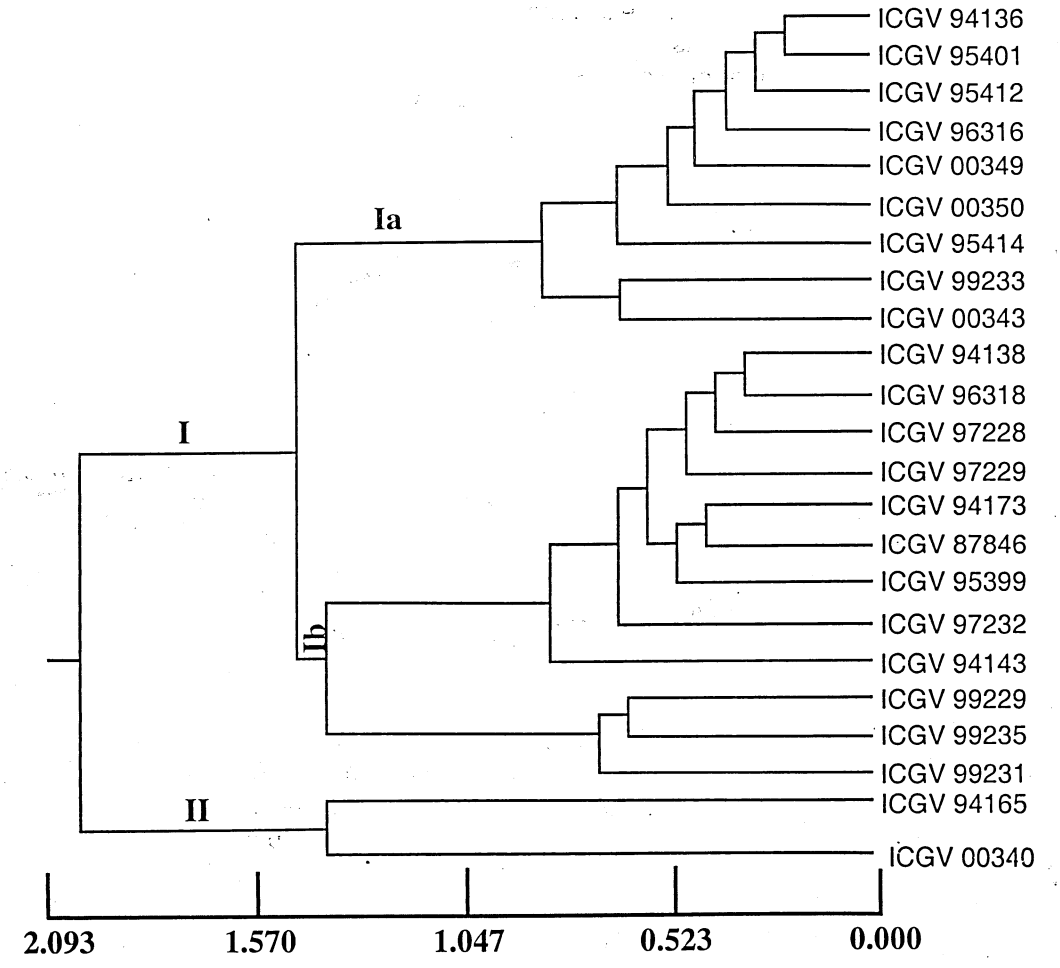

Fig. 2. UPGMA dendrogram based on Nei's (1972) genetic distance, summarizing the data on differentiation between 23 groundnut genotypes according to microsatellite marker analysis

From this study, it is evident that above groundnut genotypes were analyzed for genetic variation using SSR markers. Especially the objectives were to determine the higher genetic diversity of the groundnut genotypes as the first step for proper identification of distinct cultivars and selection of suitable parents. Finally, the identified parents will be utilized in future breeding programme of improvement of the biotic and abiotic stress tolerant groundnut varieties. Therefore, the present study assessed that there were enough scope for improvement in national breeding program by exploiting more diverse genotypes which were identified by the present study.

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