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SSR based genetic diversity analysis in a diverse germplasm of groundnut (*Arachis hypogaea* L.) from Pakistan

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Abstract

The current study was aimed to explore the genetic diversity among seventy Pakistani accessions of *Arachis hypogaea*. In Pakistan their morphological and biochemical variations have already documented but still so far, molecular variations need to be studied for this valuable crop. For molecular diversity study DNA was extracted from all seventy accessions of *Arachis hypogaea*. The extracted DNA was primed with thirty SSR primers and amplified through PCR. Fifteen out of thirty primers generated polymorphic bands among the selected accessions. In total, forty different polymorphic loci were determined across the selected accessions. The range of number of polymorphic loci detected was ranged from 2 to 4 for each primer, with an average of 2.6 loci per primer. Polymorphic Index Content (PIC) value was calculated for each marker. The dendrogram was constructed on the basis of allelic data from fifteen SSR markers across the selected accessions. All the accessions were divided into six clusters at 0.67 coefficients of similarity. This study of variations at molecular level of Pakistani groundnut accessions will be helpful for conservation and breeding purposes of groundnut and other legumes.

Keywords: Simple Sequence Repeat, Polymorphic Index Content, Groundnut, Loci.
Abbreviations: PCR_Polymerase Chain Reaction, SSR_Simple Sequences Repeat, PIC_Polymorphic Index Content.

Introduction

Groundnut (*Arachis hypogaea* L.) is grown throughout the world as a source of oil and protein. The *Arachis hypogaea* is an allotetraploid (AABB, 2n = 4x = 40 chromosomes), resulting from duplication of AA and BB wild type species (Leal-Bertioli et al., 2009). *A. hypogaea* belong to genus *Arachis* of *Fabaceae*. Two main classes of *A. hypogaea are hypogaea and fastigiata*. *A. hypogaea* have flowers on the main axis while *fastigiata* lack flowers on the main axis (Krepovickas and Gregory, 1994). The classification of groundnut only on the basis of their morphological characteristics is not sufficient therefore the assessment of variation at gene level during germplasm collections and pedigree construction is necessary. The use of molecular markers will be helpful for the collection of advanced and novel genotypes of groundnut. DNA based markers provide accurate knowledge at gene level which was not possible with phenotypic markers (Altinkut et al., 2003). Molecular markers have been used for tagging of important traits of groundnut in inter specific introgression populations. Many markers have been identified which were resistant to late leaf spot (Mace et al., 2006). Despite significant physiological, agronomic and morphological variation the peanut exhibits variations at low level by RAPD (Mondal et al., 2005), SSR (Raina et al., 2001) and AFLP (Herselman, 2003). The use of microsatellites to track advantageous traits in plant breeding and as helpful point in gene cloning program explores their importance (Brown et al., 1996). Microsatellites were mostly used in genetic diversity studies, gene flow mating system and paternity studies (Rosseto et al., 1999). Being with highest PIC, due to their high mutation rate, SSRs are routinely used in finger-printing Chen and Du, (2006), molecular mapping (Zhao et al., 2005) phylogenetic and genetic relationship studies (Yang et al., 2005) and marker-assisted breeding (Sun et al., 2006). PCR amplified the SSR loci using primers created from distinctive contiguous nucleotides. Polymorphism is due to the differences in the amount of repeats. Point mutations and polymerase slippage are the main reason of variation in number of repeats (Kruglyak et al., 1998). Microsatellites, (SSRs) have been used to study genetic variation and to construct molecular maps in numerous crops (Lee et al., 2004). ATT and CTT are the most common tri nucleotide sequences in plant genomes (Ferguson et al., 2004). Similarly AT is proved to be the most abundant dinucleotide repeat sequences followed by AG/CT and GT/CA in plant genomes (Cuc et al., 2008). The aim of the current study was to explore the genetic diversity at molecular level among accessions of *Arachis hypogea* from Pakistan. Morphological and physiological variations have been already documented but molecular variation has been not properly studied for this valuable crops. The analysis of variations at molecular level will be helpful for conservation

Table1. List of *Arachis hypogaea* accessions analyzed in this study.

No	Genotypes	Origin	No	Genotypes	Origin
1	Golden	BARI	36	Pg-686	NARC
2	Bari 2000	BARI	37	Pg-690	NARC
3	Banki	BARI	38	Pg-953	NARC
4	Chakori	BARI	39	Pg-1041	NARC
5	Bari-89	BARI	40	Pg-1044	NARC
6	Bard-479	BARI	41	Pg-1045	NARC
7	04CGOO4	BARI	42	Pg-1070	NARC
8	2KCG017	BARI	43	Pg-1074	NARC
9	2KCG020	BARI	44	Pg-1092	NARC
10	Pk-90064	BARI	45	Pg-1098	NARC
11	Icgs-09	BARI	46	Pg-1099	NARC
12	Icgs-17	BARI	47	Pg-1135	NARC
13	Icgs-18	BARI	48	Pg-1136	NARC
14	Icgs-114	BARI	49	Pg-1137	NARC
15	Icgs-38	BARI	50	Pg-1138	NARC
16	Icg-485	BARI	51	Pg-1139	NARC
17	Icg-493	BARI	52	Pg-1140	NARC
18	Icg-574	BARI	53	Pg-1141	NARC
19	Icg-2261	BARI	54	Pg-1142	NARC
20	Icg-4528	BARI	55	Bard-92	NARC
21	Icg-4747	BARI	56	Usa-7	USA
22	Icg-015662	BARI	57	M-145	USA
23	Icg-015733	BARI	58	Gs-36	AARI
24	Icg-015779	BARI	59	Gs-143	AARI
25	Yh-9307	CHINA	60	Narmal	AARI
26	Yh-14	CHINA	61	Alkat	AARI
27	Yh-11	CHINA	62	Pg-951	AARI
28	Yh-4	CHINA	63	Va-861109	AARI
29	Australian	AUSTRALIA	64	Fsd-1	AARI
30	Nigeria-10	AUSTRALIA	65	Pg-864	AARI
31	Virgina	AUSTRALIA	66	OL-598	AARI
32	Nigeria-1	AUSTRALIA	67	Icgs-10	AARI
33	NC-O6	AUSTRALIA	68	Icgs-12	AARI
34	Shi-touchi	AUSTRALIA	69	Icgs-13	AARI
35	Ncfla-14	AUSTRALIA	70	Icgs-11	AARI

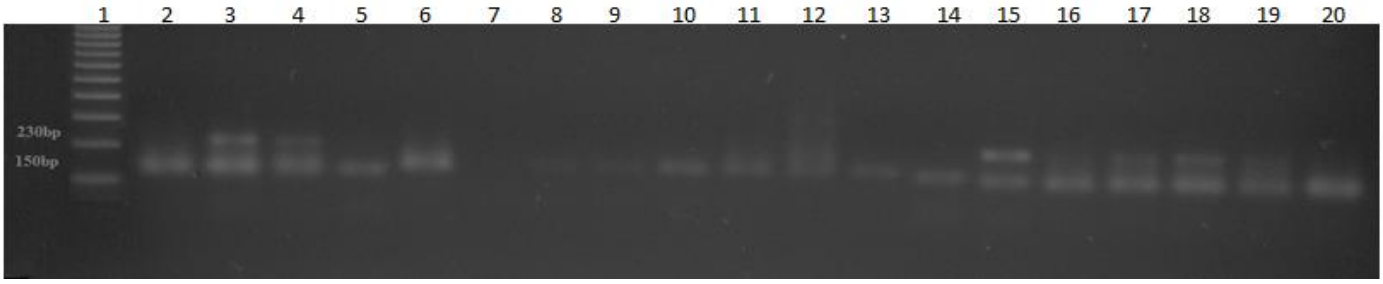

Fig 1. PCR products amplified with SSR marker Ah-10 on twenty peanut accessions.

and breeding purposes of groundnut.

Results

SSR fragment size range and polymorphism in accessions

From the initial assessment of thirty SSR markers, fifteen were selected for analysis of polymorphism among seventy groundnut accessions. A total of forty polymorphic loci were identified across seventy accessions (Table 3). Amplification with SSR primers resulted in fragment size from 75-320 base pair. Maximum fragment length (320bp) was observed in local genotypes while Australian genotype NCFLA-14 showed minimum fragment size. The number of polymorphic loci detected ranged from 2 to 5 (against each primer), with an average of 2.9 loci per primer. Among the polymorphic markers, two primers amplified five alleles each. The maximum genetic distance of 0.97 was observed between PG-1074 and USA-7 presenting them as the most divergent members. Similarly the most related members among these seventy accessions were ALKAT and ICGS-12 with 0.162 genetic distances. PIC was calculated for each marker ranged from 0.255 to 0.662 with an average of 0.548. Marker Ah-08 explored maximum diversity (PIC=0.662) while marker Ah-21 showed minimum diversity (PIC=0.255) among these 70 genotypes. Fig. 1 illustrates the result of one of the fifteen

Table 2. List of SSR markrs.

S. No	Primer	F/R	Sequences (5'-3')	Length	MW
1.	Ah-1	F	CGTTCTTTGCCGTTGATTCT	20	6.056
	Ah-1	R	AGCACGCTCGTTCTCTCATT	20	6.019
2.	Ah-2	F	GGGAATAGCGAGATACATGTCAG	23	7.162
	Ah-2	R	CAGGAGAGAAGGATTGTGCC	20	6.231
3.	Ah-3	F	AATGCATGAGCTTCCATCAA	20	6.085
	Ah-3	R	AACCCCATCTTAAAATCTTACCAA	24	7.209
4.	Ah-4	F	TGACCTCAATTTTGGGGAAG	20	6.172
	Ah-4	R	GCCACTATTCATAGCGGTA	19	5.788
5.	Ah-5	F	AAGCTGAACGAACTCAAGGC	20	6.144
	Ah-5	R	TGCAATGGGTACAATGCTAGA	21	6.494
6.	Ah-6	F	ATTCACAAGGGGACAGTTGC	20	6.166
	Ah-6	R	ATTCAAGCCTGGGAAACAGA	20	6.159
7.	Ah-7	F	TTCTTGGTTCCTTTGGCGTC	20	6.072
	Ah-7	R	TGCTCAAGTGTCCTTATTGGTG	22	6.747
8.	Ah-8	F	ATCATTGTGCTGAGGGAAGG	20	6.237
	Ah-8	R	CACCATTTTTCTTTTTCACCG	21	6.273
9.	Ah-9	F	TCAACTTTGGCTGCTTCCTT	20	6.025
	Ah-9	R	TCAACCGTTTTTCACTTCCA	20	5.978
10.	Ah-10	F	ATCACCATCAGAACGATCCC	20	6.015
	Ah-10	R	TTTGTAGCCTTCTGGCGAGT	20	6.130
11.	Ah-11	F	AAATAATGGCATACTTGTGAACAATC	26	7.986
	Ah-11	R	TTCCACCAAGGCAAGACTATG	21	6.399
12.	Ah-12	F	CTTGGAGTGGAGGGATGAAA	20	6.286
	Ah-12	R	CTCACTCACTCGCACCTAACC	21	6.231
13.	Ah-13	F	GCAAACACACCACATTTCA	19	5.710
	Ah-13	R	GGCTCCAATCCCAAACACTA	20	6.015
14.	Ah-14	F	GGGGTTCGAACGTTAATTCC	20	6.148
	Ah-14	R	CAAGAGCAACTCAATCTTCCTAGA	24	7.290
15.	Ah-15	F	TCGGAGAACCAAGCACACACATC	23	6.996
	Ah-15	R	TTGCGCTCTTTCTCACACTC	20	5.970
16.	Ah-16	F	CAGAGTCGTGATTTGTGCACTG	22	6.781
	Ah-16	R	ACAGAGTGCGCCGTCAAGTA	20	6.151
17.	Ah-17	F	CGATTTCTTTACTGAGTGAG	20	6.138
	Ah-17	R	ATTTTTTTGCTCCACACA	18	5.400
18.	Ah-18	F	ACCAAATAGGAGAGAGGGTTCT	22	6.832
	Ah-18	R	CTCTCTTGCTGGTTCTTTATTAACTC	26	7.844
19.	Ah-19	F	TTCTGATTTTAGTAGTCTTCTTTCACT	27	8.187
	Ah-19	R	CTCCTTAGCCACGGTTTCT	19	5.706
20.	Ah-20	F	TGGAATCTATTGCTCATCGGCTCTG	25	7.639
	Ah-20	R	CTCACCCATCATCATCGTTCACATT	25	7.472
21.	Ah-21	F	TCGTGTTCCCGTTGCC	17	5.128
	Ah-21	R	TCGTGTTCCCGATTGCC	20	6.101
22.	Ah-22	F	CAAGCATCAACAACAACGA	19	5.768
	Ah-22	R	GTCCGACCACATACAAGAGTT	21	6.399
23.	Ah-23	F	GAAAGAAATTATACACTCCAATTATGC	27	8.259
	Ah-23	R	CGGCATGACAGCTCTATGTT	20	6.108
24.	Ah-24	F	CCTTTTCTAACACATTCACACATGA	25	7.520
	Ah-24	R	GGCTCCCTTCGATGATGAC	19	5.780
25.	Ah-25	F	AGTGTTGGGTGTGAAAGTGG	20	6.308
	Ah-25	R	GGGACTGGGAACAGTGTTTATC	22	6.830
26.	Ah-26	F	TGTGAAACCAAATCACTTTCATTC	24	7.271
	Ah-26	R	TGGTGAAAAGAAAGGGGAAA	20	6.312
27.	Ah-27	F	ACTCGCCATAGCCAACAAAC	20	6.024
	Ah-27	R	CATTCCCACAACTCCCACAT	20	5.926
28.	Ah-28	F	CAATTCATGATAGTATTTTATTGGACA	27	8.287
	Ah-28	R	CTTTCTCCTCCCCAATTTGA	20	5.954
29.	Ah-29	F	CCTATCCTATGGGTCACTAGCC	22	6.646
	Ah-29	R	GCTTGTGCTCATCTTGAGTTTT	22	6.713
30.	Ah-30	F	AGTGTTGGGTGTGAAAGTGG	20	6.308
	Ah-30	R	GGGACTCGGAACAGTGTTTATC	22	6.790

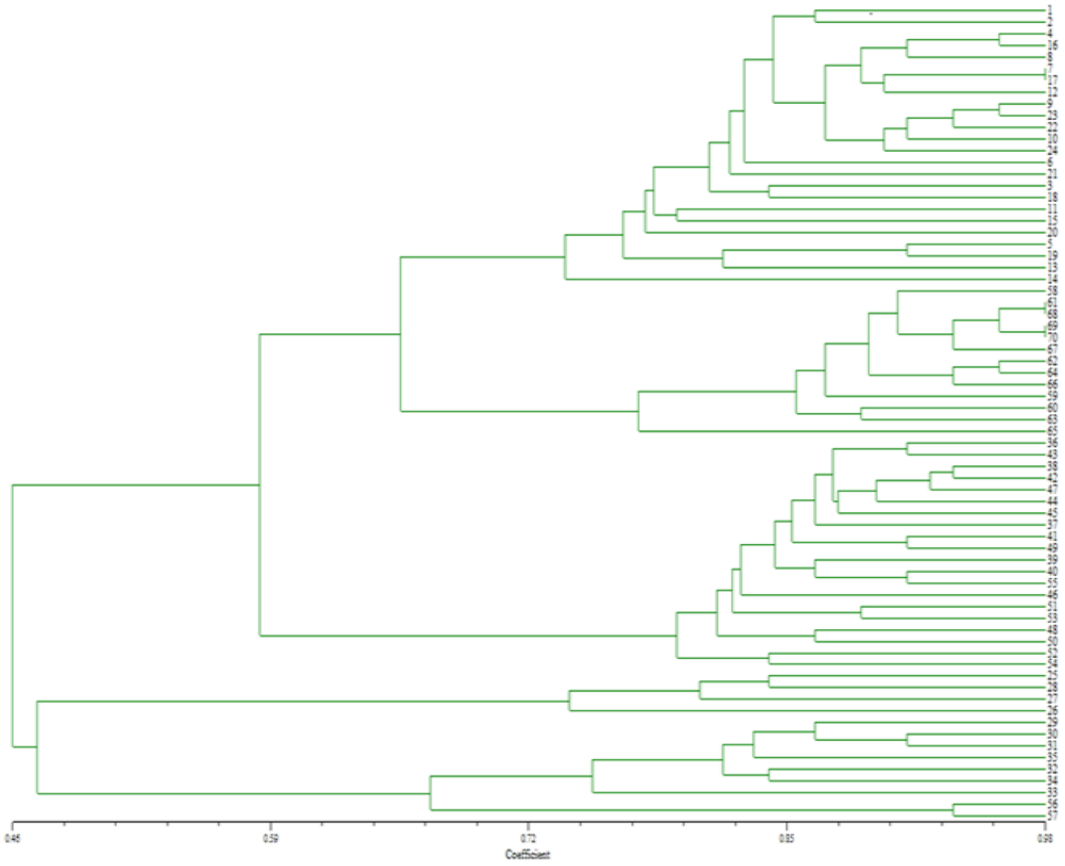

Fig. 2 Relationships among seventy *Arachis hypogea* accessions based on amplification events obtained using fifteen SSR primers. All the accessions were divided into six clusters at 0.67 coefficients of similarity.

SSR primers. The individual primers gave different banding pattern. In this study, not all markers clearly showed definite amplification fragments. Some markers showed no amplification band within several genotypes while showing clear and bright amplification bands in other genotypes. For example the primer Ah-26 and Ah-08 showed clear amplification products in all genotypes except Banki, Chakori and Bari-89 genotypes suggesting the deletion of primer binding site or complete lack of the primer locus in these genotypes. Few markers were noted to be variety-specific producing different alleles in different genotypes. Primer Ah-27 amplified five alleles in Icgs-38, three in Yh-11 and two in the other genotypes.

Cluster analysis

The dendrogram was constructed on the basis of allelic data from fifteen SSR markers across the selected accessions as shown in Fig. 2. At 0.67 coefficients of similarity all the accessions were grouped into six clusters. The selected accessions were grouped mainly according to their breeding /research stations. These clusters were further divided into sub clusters. The cluster A contains 24 genotypes evolved at BARI. These varieties showed relatively high similarity with one other. Genotypes ICGS-493 and ICGS-38 showed maximum similarity and the genotype ICGS-14 is the most divergent genotype of this group. Similarly the cluster B contains 13 genotypes evolved at AARI. The genotypes of this group showed maximum similarity with each other when compared with the rest of genotypes. Genotypes ICGS-12 and ALKAT showed maximum similarity while PG-864 is the most divergent genotype of this group. Cluster C consists of 20 genotypes developed at NARC. PG-1142 is the most diverse member of this group. In this group the two genotypes PG-953 and PG-1070 are the most similar members. Cluster D contains only 4 genotypes. All the genotypes are of Chinese origin. The genotypes of this group showed minimum similarity with each other when compared with the rest of genotypes. YH-14 is the most divergent member of the group. The cluster E contains 10 genotypes of Australian origin while the cluster F has only two genotypes of USA origin. Eight genotypes of Sub-group E showed relatively less similarity with each other as compared to sub group F. Pakistani origin accessions group together at 0.59 co-efficient and are much diverse from each other while Chinese and US origin accessions were group together at 0.75 co-efficient.

Discussion

The identification of agronomically valuable and diverse cultivars at molecular level will be very useful for linkage mapping and genetic improvement of specific traits in groundnut. Microsatellites have widely used as molecular markers in recent years. Among the DNA markers, SSR markers are more preferable as it is highly abundant, genetically co-dominant, analytically simple and are highly reproducible. The first report of microsatellites in plants was reported by Condit and Hubbel, 1991, who reported that SSR markers are abundant in plants. The report SSR polymorphisms on soybean by Akkaya et al., 1992 have opened up a novel source of PCR-based DNA markers for other crops. The high polymorphism and stability of SSR markers made them useful and superior in molecular genetic

Table 3. Results of the fifteen SSR markers used in PCR.

Primer	F/R	Sequence (5'-3')	Length	MP	PIC	No of Alleles	Fragment bp
Ah-5	F	AAGCTGAACGAACTCAAGGC	20	58	0.498	2	260
	R	TGCAATGGGTACAATGCTAGA	21				
Ah-8	F	ATCATTGTGCTGAGGGAAGG	20	58	0.662	3	170
	R	CACCATTTTTCTTTTTCACCG	21				
Ah-9	F	TCAACTTTGGCTGCTTCCTT	20	58	0.626	3	120
	R	TCAACCGTTTTTCACTTCCA	20				
Ah-10	F	ATCACCATCAGAACGATCCC	20	56	0.641	4	200
	R	TTTGTAGCCTTCTGGCGAGT	20				
Ah-12	F	CTTGGAGTGGAGGGATGAAA	20	56	0.457	2	285
	R	CTCACTCACTCGCACCTAACC	21				
Ah-13	F	GCAAACACACCACATTTCA	19	58	0.547	2	160
	R	GGCTCCAATCCCAAACACTA	20				
Ah-15	F	TCGGAGAACCAAGCACACACATC	23	56	0.636	2	290
	R	TTGCGCTCTTTCTCACACTC	20				
Ah-16	F	CAGAGTCGTGATTTGTGCACTG	22	58	0.61	4	320
	R	ACAGAGTGCGCCGTCAAGTA	20				
Ah-19	F	TTCTGATTTTAGTAGTCTTCTTTCACT	27	58	0.595	3	195
	R	CTCCTTAGCCACGGTTTCT	19				
Ah-20	F	TGGAATCTATTGCTCATCGGCTCTG	25	56	0.608	3	200
	R	CTCACCCATCATCATCGTTCACATT	25				
Ah-21	F	TCGTGTTCCCGTTGCC	17	58	0.255	2	145
	R	TCGTGTTCCCGATTGCC	20				
Ah-23	F	GAAAGAAATTATACACTCCAATTATGC	27	58	0.503	2	190
	R	CGGCATGACAGCTCTATGTT	20				
Ah-25	F	AGTGTTGGGTGTGAAAGTGG	20	58	0.561	3	240
	R	GGGACTGGGAACAGTGTTTATC	22				
Ah-27	F	ACTCGCCATAGCCAACAAAC	20	56	0.563	2	210
	R	CATTCCCACAACTCCCACAT	20				
Ah-30	F	AGTGTTGGGTGTGAAAGTGG	20	58	0.456	3	200

studies and varietal identification (Varshney et al., 2005).The present study demonstrates the presence of significant polymorphic SSR markers in *A. hypogaea*. Fifty percent polymorphism was identified among the tested genotypes in the current research work. The results revealed the presence of significant level of polymorphism in cultivated peanut. Several other studies have also reported a significant level of polymorphism in cultivated peanut (Mace et al., 2007) observed a high level of polymorphism within germplasm of peanut which are resistant to bacterial wilt. He reported that 99.4% of the genotypes showed polymorphism when screened with thirty two SSR primers. Similarly 76.5% polymorphism was described by Mondal S and Badigannavar AM (2010) when they studied the connection of simple sequence repeat (SSR) with late leaf spot and rust resistance in groundnut. The study of (Cuc et al., 2008) in which 44% polymorphism was observed, were similar to our results In contrast to our work various previous works have resulted in a low level of polymorphism among cultivated peanut genotypes (Halward et al., 1991; He and Prakash, 1997; Hopkins et al., 1999; Herselman, 2003; Moretzsohn et al., 2004; Mace et al., 2006). Insignificant molecular diversity among wild and cultivated peanut genotypes was detected by using SSR markers (Hopkins et al., 1999, Krishna et al., 2004, He et al., 2005). The insignificant molecular diversity among the parental lines might be the reason of low level of polymorphism in cultivated peanut. The origination of cultivated groundnut from a single polyploidization practice is the main reason of low level of genetic polymorphism (Young et al., 1996). In the current study the PIC value was calculated for each marker ranged from 0.255 to 0.662 with an average of 0.548. The study of (Cuc et al., 2008) in which an average of 0.46 PIC was calculated, are in support of our findings. Similarly (Geleta et al., 2006) observe an average of 0.64 PIC value for SSR markers among 45 accessions of groundnut. The number of alleles per marker detected in this study, ranged from 2 to 4 with an average of 2.9 alleles per locus matched with the earlier studies. (Cuc et al., 2008) showed similar results of 2 to 5 alleles with an average of 2.9 alleles per locus in 32 genotypes of groundnut. Our results are in pipeline with the finding of (Tang et al., 2007) in which 2.0-5.5 alleles per locus for were observed. The work of (Hopkins et al., 1999) also favor multiloci concept of SSR markers. The main reasons behind the contrary results of molecular markers might be due to the different genetic sources of peanut germplasm and primer variation in quality and quantity. In short it is summarized that molecular level genetic diversity is superior as compared to morphological and biochemical polymorphism. Furthermore the assessment of molecular level diversity is more important for preservation of genetic assets, recognition of best germplasm resources and the collection of superior cultivars for hybridization purposes (Dwived et al., 2001). The results from the genetic similarity-based analysis of seventy groundnut accessions with fifteen polymorphic microsatellite markers prominently differentiate the local varieties into their breeding/research station. The clustering of the same institutions accessions into one major group is consistent with conventional taxonomical classification of cultivated peanut (Tang et al., 2008). One possible reason for this close association could be the involvement of common parents/lines in the development of these varieties. Thus the enhancement of diverse populations by using diverse germplasm resources is needed in the future for various purposes such as population genetic structure, germplasm analysis, identification of cultivars, selection of parents and phylogenetic relationships.

Materials and methods

Plant materials

A total of seventy accessions of groundnut were collected from different research institute such as NARC, BARI and AARI for the current study (Table 1) which consists of four Chinese, two USA, seven Australian and fifty seven of Pakistani origin accessions. The research work was carried out at National Institute for Genomics and Advanced Biotechnology (NIGAB), NARC, Islamabad in 2012.

DNA extraction

DNA was extracted from the leaves of seventy accessions of groundnut through a modified CTAB-based procedure (Mace et al., 2003). The quality of DNA was measured by running DNA on 1% agarose gel and quantified with spectrophotometer at wavelength of 260 nm.

PCR analysis

Thirty different SSR markers were used in the current study (Table 2). PCR was carried out in Veriti 96-well thermal cycler (Applied Biosystems, CA) with Taq polymerase (MBI Fermentas). DNA samples were diluted to 20ng/µl for PCR. PCR reaction were performed in 20 µl volumes PCR mixture, containing 30 ng/µl genomic DNA, IX PCR buffer (MBI Fermentas) 1.6 mM MgCl2, 0.5 mM dNTPs, 0.5 U DNA polymerase (MBI Fermentas) and 10 p mol of each primer. The initial denaturation temperature of 94ºC for 04 min was adjusted followed by the 40 cycle of Denaturation (94 for 45sec), annealing (58ºC for 60 sec) and Extension (72ºC for 90 sec). The quality and concentration of extracted DNA was assessed by visualizing it on 2% agarose gel under UV light.

Data analysis

The presence/absence of each fragment (product length variant) and banding pattern in each accession was recorded against each primer. The size of the amplified product was calculated on the basis of its mobility relative to molecular mass of marker (100bp, MBI, Fermentas). Pair-wise comparisons of the cultivars based on the ratio of exceptional to common alleles were used to calculate the genetic similarity by Dice coefficients using SIMQUAL sub-program in similarity routine of software NTSYS-pc version 2.2 (Exeter Software, Setauket, NY, U.S.A.) software package (Rohlf, 2005). Estimates of genetic similarity were considered among all pairs of the genotypes according to Nei and Li (1979). Dendrogram was constructed using UPGMA to calculate genetic associations among peanut accessions by the protocol earlier described by Rohlf, 2005. The PIC for each SSR was determined according to Anderson et al. (1993).

Conclusion

The results showed that fifteen out of thirty markers produced a total of forty loci across Pakistani accessions of groundnut. The number of polymorphic loci detected ranged from 2 to 4 (against each primer, with an average of 2.6 loci per primer. Marker Ah-20 showed maximum diversity (PIC=.663) while marker Ah-21 showed minimum diversity (PIC=.255) among these 70 genotypes. At 0.59 coefficient of similarity all the seventy genotypes were divided into four major groups according to their breeding/research stations. The current study highlights the need of isolation and characterization of more DNA markers in groundnut and their uses in advanced studies such as gene discovery, marker assisted selection and gene mapping. SSR markers used in this study can also be used in other species as well. It will reduce the cost of the study, since microsatellite markers improvement is still costly and sustained.

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