Electronic Journal of Plant Breeding, 1(6): 1420-1425 (Dec 2010)
ISSN 0975-928X

Research Article

Genetic diversity analysis of groundnut genotypes using SSR markers

D. Shoba, N. Manivannan and P.Vindhiyavarman

Dept. of Oilseeds, Tamil Nadu Agricultural University, Coimbatore
Email: shobashoba7@gmail.com

(Received: 27 Aug 2010; Accepted:26 Oct 2010)

Abstract:
Groundnut (*Arachis hypogaea* L.), an important oilseed crop is a rich source of oil and protein. Molecular marker technologies are the effective tools and they are used for the assessment of genetic variability because they are not influenced by the environment. Among the molecular markers, Simple Sequence Repeat (SSR) has proved to be the most powerful tool for variety identification in groundnut and has much potential in genetic and breeding studies. Among the 17 SSR primer pairs used for assessing the genetic diversity, 6 primer pairs (24.0 per cent) were polymorphic. The genotype TMV 2 was susceptible to rust and late leaf spot diseases and it was separately clustered in the dendrogram and among the eleven foliar disease resistant genotypes, the genotypes *viz.,* COG 0423, COG 0436 and COG 0432 were distantly clustered from TMV 2. Hence, by using the genotypes *viz.,* TMV 2, COG 0423, COG 0436 and COG 0432, three combinations *viz.,* TMV 2 x COG 0423, TMV 2 x COG 0436 and TMV 2 x C0G 0432 could be made for further studies.

Key words: Groundnut, SSR markers, DNA polymorphism, genetic diversity.

Introduction

Groundnut (*Arachis hypogaea* L.) is an important crop among oilseeds, as it can be consumed and utilized in diverse ways. Groundnut seeds are rich in oil and protein. In India, it occupies an area of 6.41 million ha with a production of 9.36 million tonnes, which accounts for a productivity of 1460 kg/ha during 2007-08 (Anonymous, 2008). In India, 70 per cent of the groundnut area and 75 per cent of the production are concentrated in the four states of Gujarat, Tamil Nadu, Andra Pradesh and Karnataka. In Tamil Nadu, the area under groundnut is about 0.51 million ha, with a production of 1.01 million tonnes and the productivity of 1981 kg/ha during 2007-08 (Anonymous, 2008). It is difficult to classify the accessions solely based on their morphological characters. The development of reliable methods is necessary to allow for the assessment of genetic variability in germplasm collections or pedigree reconstruction. In various methodologies, DNA based technologies are the most reliable tools allowing for the assessment of genetic variability because they are not influenced by the environment. DNA markers *viz.*, RFLP, RAPD, SSR, ISSR *etc*. can be used to assess the diversity studies. Among them, SSR markers have great potential in genetic and breeding studies. The objectives of the present study are to use SSR markers to detect DNA polymorphism among cultivated groundnut genotypes and for selecting parents for further breeding programmes.

Material and Methods

Eleven groundnut genotypes consisting of ten late leaf spot and rust resistant genotypes *viz.,* COG 0417, C0G 0422, COG 0423, COG 0432, COG 0436, COG 0437, COG 0438, COG 0440, ICGV 97150 and ICGV 97163 and one susceptible genotype TMV 2 were used in the present study. The characteristic features of these genotypes are presented in Table 1.

DNA extraction

Genomic DNA of all the 11 parents was extracted by Cetyl Trimethyl Ammonium bromide (CTAB) method (Doyle and Doyle, 1987). The collected leaf samples of 10-15 days old plants were ground in pestle and mortar by using preheated (around 65ºC) CTAB (For 100 ml: 1 M Tris(pH-8.0)- 10 ml, 5 M NaCl- 14 ml, 0.5 M EDTA(pH-8.0)-2 ml, 2-Mercapto ethanol-1 ml, CTAB-2g and dH_20-73 ml) buffer. Around 450 µl of CTAB buffer was added. Extracted samples were taken into eppendorf tubes and incubated in a water bath for 30 min at 65º C. After incubation, around 450 µl of chloroform: isoamyl alcohol (24:1) was added into the tubes and inverted twice to mix. Then the tubes were kept in centrifuge for 10 minutes at 5500 rpm. Then the aqueous layer was transferred in to new epperndorf tubes. An amount of 0.7 volume of iso propanol (stored at -20^{0}C) was added to each sample

and inverted once to mix and kept overnight at 4^0C. The samples were centrifuged at 5500 rpm for 15 min on the next day. The supernatant was discarded from each sample and the pellets settled in the bottom were air dried for 30 min. A quantity of 100 µl of TE buffer was added into each sample and stored overnight at 4^0C. RNAse (3 µl) was added into each sample to exclude the RNA contamination on the following day. An amount of 200 µl chloroform: isoamyl alcohol (24:1) was added into the tubes and centrifuged at 5000 rpm for 5 minutes and the supernatant was taken into the fresh tubes to which twice the volume of absolute ethanol and $1/10^{th}$ volume of 3M sodium acetate added and kept at -20^0C for 5 minutes and again centrifuged at 5500 rpm for 15 minutes. The supernatant was discarded and 200 µl of 70 per cent ethanol was added and centrifuged at 5000 rpm for 5 minutes. The supernatant was discarded and the pellet was air dried for 30 minutes. The pellet was resuspended by using 100 µl TE buffer and kept at -20□ C for long term use.

SSR analysis

Seventeen SSR markers were used for analyzing 11 groundnut genotypes. The polymerase chain reaction (PCR) mixtures (10 µl) contained 2 µl template DNA (5 ng), 1 µl of 10 X Taq buffer + $MgCl_2$ (15mM), 1 µl of dNTP (2 mM), 0.5 µl of primers 10 µ M (Forward and Reverse), 0.1 µl of taq polymerase (Genei 5µ/µl) and 4.9 µl of sterile double distilled water. Amplification was performed in 0.2 ml (each tube) thin walled PCR plates (96 tubes / plate) in a thermal cycler (Applied Biosystems). The samples were initially incubated at 94.0°C for 3 min and then subjected to two times 19 repeats of the following cycle: 94.0 °C for 30 Sec (-0.5^0C for every cycle), 63.0°C for 30 sec, 72.0°C for 1 min; another 19 cycle: 94.0°C for 15 sec, 55.0°C for 30 sec, 72.0°C for 1 min. Final Extension is 72.0°C for 10 min. Loading dye (100 ml 10 X loading dye):Glycerol-50 ml, bromophenol blue- 0.4 g, xylene cyanol- 0.4 g, ultra pure water-50 ml) was added into PCR products before electrophoresis. Amplified products were analyzed using 6% non denaturing polyacrylamide gel (100 ml gel recipe: 20 ml of acrylamide/bisacrylamide 29:1 (w/w), 10 ml of 10X TBE,70.5 ml distilled water, 900 µl Ammonium persulphate, TEMED 200 µl). Twenty base pair ladder was used to assess the base pair difference between 11 genotypes. Electrophoresis was performed at constant power 400 volts for about 4 h and silver stained (Benbouza *et al.* 2006).

Analysis of genetic diversity

Genetic diversity among the 11 groundnut genotypes were evaluated using 17 SSR primers. Each fragment size was treated as a unique characteristic and scored as present (1) or absent (0). Genetic similarity index (UPGMA cluster analysis of the Jaccard's similarity coefficient) was used to construct a dendrogram which illustrated the genetic relationship among the eleven genotypes of groundnut used in the study. A dendrogram was constructed using similarity index adopting Sequential Hierarchial and Nested (SAHN) using the NTSYS program (Rohlf, 2000).

Results and Discussion

In any plant breeding programme, assessment of parental divergence is an important and foremost objective. The threat to genetic erosion has led to a significant interest in the assessment of genetic diversity in germplasm collections (Manifesto *et al*., 2001). It helps in identifying the desirable parents for hybridization programme. Molecular markers are useful complement to morphological and physiological characterization of cultivars because they are plentiful, independent of tissue or environmental effects and allow cultivar identification early in plant development. The molecular markers based on differences in DNA sequences between individuals generally detect more polymorphisms than morphological and protein based markers (Mignouna *et al*., 1998; Tanksley *et al*., 1989). Simple Sequence Repeats (SSR) is used as a primer to amplify regions between the microsatellites. This marker reveals a much larger number of fragments per primer than RAPD analysis (Bajpai *et al*., 2008).

Polymorphism of SSR markers

In order to analyse the extent of genetic diversity among the 11 genotypes of groundnut, 17 SSR primer pairs were used. The sequence of all the 17 SSR primers is given in Table 2. Out of 17 SSR primer pairs used for assessing the genetic diversity, 6 primer pairs (24.0 per cent) were polymorphic. The number of bands produced by the primers ranged from 1 to 3. The bands were scored in binaries as presence (1) or absence (0). The pictorial representation of all the 11 genotypes for the SSR primer 210 has been given in Fig 1. The Polymorphic Information Content (PIC) value was calculated to estimate the informativeness of each primer and it varied from 0.17 to 0.63 with an average of 0.41 (Table 3).

Cluster analysis

Genetic similarity co-efficient estimate for the 11 genotypes ranged from 0.54 to 1.00 (Table 4). Based on similarity values, genotypes COG 0423 and COG 0438 had less similarity and genotypes COG

0417, COG 0422, COG 0437 had high similarity. Based on the dendrogram (Fig 2), 11 genotypes of groundnut were grouped into six clusters at 0.92 coefficient. Among the six clusters, genotypes TMV 2, COG 0423, COG 0432 and COG 0436 were clustered in four separate clusters *viz.*, I, IV, V and VI. Habit of the two genotypes *viz.*, TMV 2 and COG 0423 were Spanish bunch; while all other genotypes belong to Virginia bunch. The second cluster included four genotypes such as COG 0417, COG 0422, COG 0437 and COG 0438. The third cluster included three genotypes such as COG 0440, ICGV 97163 and ICGV 97150. As mentioned earlier, TMV 2 was susceptible to foliar diseases and it was separately clustered in the dendrogram. Among the eleven foliar disease resistant genotypes, COG 0423, COG 0436 and COG 0432 were distantly clustered from TMV 2. Hence, by using the genotypes *viz.,* TMV 2, COG 0423, COG 0436 and COG 0432, three combinations *viz.,* TMV 2 x COG 0423, TMV 2 x COG 0436 and TMV 2 x C0G 0432 could be made for further studies.

Genetic diversity studies in cultivated groundnut using SSR markers were reported by various authors. Twenty three SSRs were screened across 22 groundnut genotypes with differing levels of resistance to rust and LLS by Mace *et al*. (2006) and they reported that twelve of the 23 SSRs (52 per cent) showed a high level of polymorphism with PIC values ≥0.5. Thirty-four SSR markers were used to assess the genetic variation of four sets of twenty-four accessions each from the four botanical varieties of the cultivated peanut were as reported by Tang *et al.* (2007). In their report, among the tested accessions, ten to sixteen pairs of SSR primers showed polymorphisms and the dendrograms based on genetic distances were constructed for the four botanical varieties, which revealed the existence of different clusters. Finally, they concluded that there was abundant intra-variety SSR polymorphism, and with more and more SSR markers being developed, the intrinsic genetic diversity would be detected and the development of genetic map and marker-assisted selection for cultivated peanut would be feasible. Molecular diversity and association of simple sequence repeat (SSR) markers with rust and late leaf spot (LLS) resistance were detected in a set of 20 cultivated groundnut genotypes differing in resistance against both diseases and were reported by Mondal and Badigannavar (2009). In their report, out of 136 bands amplified from 26 primers, 104 were found polymorphic (76.5 per cent). Cluster analysis (UPGMA) revealed two main clusters separated at 52 per cent Jaccard's similarity coefficient according to disease reaction to rust and LLS.

References

Anonymous, 2008. Agricultural Statistics at a Glance-2007-08. Directorate of Economics and Stastistics, Ministry of Agriculture, Govt. of India, Krishi Bhavan, New Delhi.

Bajpai, A., Srivastava, N., Rajan, S and Chandra, R. (2008). Genetic diversity and discrimination of mango accessions using RAPD and ISSR markers. *Indian J. Hort.,* **65**(4)**:** 377- 382.

Benbouza, H., Jacquemin, J.M., Baudoin, J.P and Mergeai, G. (2006). Optimization of a reliable, fast, cheap and sensitive silver staining method to detect SSR markers in polyacrylamide gels. *Biotechnol. Agron. Soc. Environ.,* **10**(2):77-81.

Doyle, J.J and Doyle, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull.,* 19**:** 11-15.

Mace, E.S., Phong, D.T., Upadhaya, H.D., Chandra, S and Crouch, J.H. (2006). SSR analysis of cultivated groundnut (*Arachis hypogaea* L.) germplasm resistant to rust and late leaf spot diseases. *Euphytica, 152* (3) :317-330.

Manifesto, M. M., Schlatter, A. R., Hopp, H. E., Suarez, E. Y and Dubcovsky, J. (2001). Quantitative evaluation of genetic diversity in wheat germplasm using molecular markers. *Crop Sci.,* 41**:** 682-690.

Mignouna, H. D., Ikca, N. Q and Thottapilly, G. (1998). Genetic diversity in cowpea as revealed by random amplified polymorphic DNA. *J. Genet. Breed.,* **52**: 151-159.

Mondal, S and Badigannavar, A.M. 2009. Molecular diversity and association of SSR markers to rust and late leaf spot resistance in cultivated groundnut (*Arachis hypogaea* L.). *Plant Breed.,* **129**(1): 68-71.

Rohlf, J. (2000). NTSYSpc: Numerical taxonomy and multivariate analysis system. Version 2.1. *Users Guide, Exeter Software, Setauket***,** 38, New York.

Tang, R., Gao, G., He, L., Han, Z., Shan, S., Zhong, R., Zhou, C., Jiang, J., Li, Y and Zhuang, W. 2007. Genetic diversity in cultivated groundnut based on SSR markers. *J. Genet. and Genomics,* **34**(5): 449-459.

Tanksley, S. D., Young, N. D., Paterson, A. H and Bonierbale, M. W. (1989). RFLP mapping in plant breeding: New tools for an old science. *Biotechnol.,* **7:** 257-264.

Table 1. Particulars of parents studied

Genotypes	Pedigree	Habit
TMV 2	Mass selection from Gudiatham Bunch(AH 32)	Spanish bunch
COG 0417	TMV 2 x ICGV 96266	Virginia bunch
COG 0422	TMV 7 x ICGV 94118	Virginia bunch
COG 0423	TMV 2 x ICGV 96266	Spanish bunch
COG 0432	TMV 7 x ICGV 96275	Virginia bunch
COG 0436	TMV 7 x ICGV 96275	Virginia bunch
COG 0437	CO 2 x ICGV 94118	Virginia bunch
COG 0438	CO 2 x ALR 2	Virginia bunch
COG 0440	GG 2 x ICGV 94118	Virginia bunch
ICGV 97150	(JH 60 x PI 259747) x NCAC 343) x ICGV 86003)	Virginia bunch
ICGV 97163	ICGV 88268 x ICG 7707	Virginia bunch

Table 2. Sequence of SSR primers used

Primers	Forward sequence	Reverse sequence
PGS17H05	TCGTAAGTTCAACCTCGGCT	AATGGCGTCGTTTGATTCAT
PM 210	CCGCAGATCTTCTCCTGTGT	CCTCCTCATCCTCTAAACTCTGC
PM 3	GAAAGAAATTATACACTCCAATTATCG	CGGCATGACAGTCCTATGTT
PM 305	GCGCTGGAACACAGTAAGAC	GGCAGAAGGAAAGTTGCAG
PM 325	CCTAACAAGGACGGGTGAAC	CAGAGGCCTCACTTTCCTTC
PM 36	ACTCGCCATAGCCAACAAC	CATTCCCACAACTCCCACAT
Ah 41	CGCCACAAGATTAACAAGCACC	GCTGGATCATTGTAGGGAAGG
PGP02D12	AAGCTGAACGAACTCAAGGC	TGCAATGGGTACAATGCTAGA
PGP08D09	TGAGTTTCCCCAAAAGGAGA	CAACAACAATACGGCAACA
PGS13A07	AATCCCACGCAATGATAAAAA	TCCCCTTATTGTTCCAGCAG
PGS13C08	GATACAGCATTTTGGGCCTC	AAAGCTCATGAAAGCCGAGA
PGS15D03	CATGCCATCATCACAACACA	GGAGGAAGCAATGGTTTCAG
PM 375	CGGCAACAGTTTTGATGGTT	GAAAAATATGCCGCCGTTG
PM 45	TGAGTTGTGACGGCTTGTGT	GATGCATGTTTAGCACACTTGA
PMC 297	ATGCACCTGCAAGTGAAGAC	TCAAGGATGCAGCAAGACAC
PM 15	CCTTTTCTAACACATTCACACATGA	GGCTCCTTCGATGATGAC
PM 32	AGTGTTGGGTGTGAAAGTGG	GGGACTCGAAACAGTGTTATC

Table 3. Percentage of polymorphism and Polymorphic Information Content (PIC) value for the SSR primers

Primers	Total no. of bands	No. of polymorphic bands	PIC value
PGS17H05	1	-	0.00
PM 210	3	2	0.46
PM 3	2	1	0.63
PM 305	1	-	0.00
PM 325	2	-	0.00
PM 36	2	1	0.40
Ah 41	1	-	0.00
PGP02D12	1	-	0.00
PGP08D09	1	-	0.00
PGS13A07	1	-	0.00
PGS13C08	1	-	0.00
PGS15D03	2	2	0.17
PM 375	2	2	0.30
PM 45	1	-	0.00
PMC 297	3	1	0.50
PM 15	2	-	0.00
PM 32	1	-	0.00

Table 4. Genetic similarity co-efficient values between 11 groundnut genotypes

	TMV2	COG 0417	COG 0422	COG 0423	COG 0432	COG 0436	COG 0437	COG 0438	COG 0440	ICGV 97150	ICGV 97163
TMV2	1.00										
COG 0417	0.69	1.00									
COG 0422	0.69	1.00	1.00								
COG 0423	0.69	0.57	0.57	1.00							
COG 0432	0.71	0.78	0.78	0.80	1.00						
COG 0436	0.78	0.86	0.86	0.73	0.90	1.00					
COG 0437	0.69	1.00	1.00	0.57	0.78	0.86	1.00				
COG 0438	0.65	0.95	0.95	0.54	0.83	0.83	0.95	1.00			
COG 0440	0.73	0.88	0.88	0.57	0.83	0.83	0.88	0.92	1.00		
ICGV 97150	0.73	0.88	0.88	0.57	0.83	0.83	0.88	0.92	0.92	1.00	
ICGV 97163	0.78	0.85	0.85	0.56	0.80	0.80	0.85	0.88	0.96	0.96	1.00

Fig. 1 The pictorial representation of all the 11 genotypes for the SSR primer 210

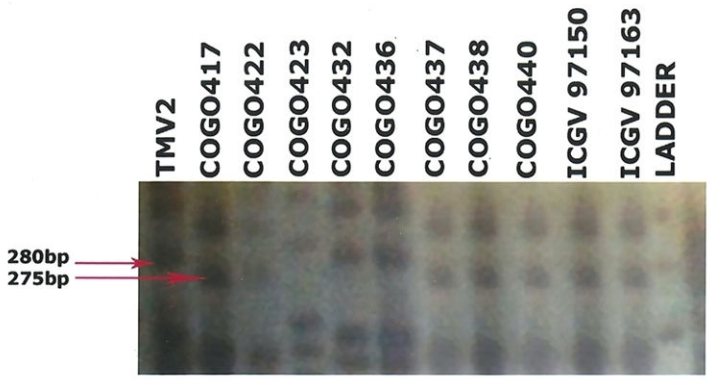

Fig. 2 Dendrogram of 11 genotypes of groundnut based on Jacard's similarity Coefficient