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Assessment of genetic diversity in cultivated groundnut (*Arachis hypogaea* L.) with differential responses to rust and late leaf spot using ISSR markers

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Abstract

Inter simple sequence repeat (ISSR) markers were used to analyze genetic diversity among twenty cultivated groundnut (*Arachis hypogaea* L.) genotypes. Out of 154 amplicons produced by 21 ISSR primers, 115 were found polymorphic (74.67%). The 3'-anchored primers based on poly 'GA' and poly 'AG' motifs produced higher proportion of polymorphism of 74.85% and 77.27%, respectively. The primer UBC 810 identified 18 genotypes out of 20 studied as it had the highest resolving power among the 21 primers. The dendrogram obtained from Ward method of cluster analysis revealed four subclusters, of which subcluster A and subcluster D contained resistant and susceptible genotypes, respectively. Based on Kruskal-Wallis one-way ANOVA, UBC 810_{540} was found associated with both rust and late leaf spot (LLS) resistance and UBC 810_{500} with LLS resistance.

Key words: Groundnut, foliar diseases, ISSR markers, PIC, resolving power

Introduction

Groundnut (*Arachis hypogaea* L.), commonly known as peanut, is an important legume and oilseed crop, which originated in Bolivian region (South Bolivia-North Western Argentina) [1]. It is being cultivated in different agro-climatic conditions in many states in India. Around 80% of groundnut area is rainfed, wherein the foliar diseases such as rust (*Puccinia arachidis* Speg.) and late leaf spot (LLS) (*Phaeoisariopsis personata* Berk & Curt.) are very much prevalent and cause yield losses up to 50% [2]. Although, sufficient resistant source for rust is available but very limited sources of resistance exist for LLS within *A. hypogaea* gene pool. Earlier efforts, made to introgress resistant genes from wild *Arachis* species to cultivated groundnut, have had limited success due to the linkage drag of undesirable pod trait like poor shelling outturn and prominent reticulation and deep constriction in the pods with disease resistance [3]. In this regard, major emphasis was directed towards crosses within diverse cultivated groundnut genotypes differing in resistance against rust and LLS. However, this attempt needs proper documentation of genetic diversity among cultivated groundnut genotypes differing in their resistance level against both the diseases. Evaluation of genetic diversity based on morphological features may not be efficient as they are highly influenced by environments. To overcome these problems, biochemical and molecular techniques would be of great help. Earlier, limited genetic variation had been reported from evaluation of seed proteins [4]. Further, molecular marker systems such as, Restriction Fragment Length Polymorphism (RFLP) [5], Randomly Amplified Polymorphic DNA (RAPD) [6, 7] and Amplified Fragment Length Polymorphism (AFLP) [8] detected low level of genetic diversity among cultivated groundnut genotypes. Inter simple sequence repeat (ISSR) marker technique is a PCR based method, which involves amplification of DNA present at an amplifiable distance in between two identical microsatellite repeats oriented in opposite direction [9]. The technique uses microsatellites, usually 16-25 bp long as primers in a single primer PCR reaction targeting multiple genomic loci to amplify the inter simple repeat sequences of different sizes. ISSR's have high reproducibility possibly due to the use of longer primers (16-25 bases) as compared to RAPD primers (decamers) that permits the subsequent use of high annealing temperature (45-60^{o}C) leading to higher stringency. Such DNA markers are considered best tool

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for determining genetic relationship/diversity as they are abundant in number, highly polymorphic and are independent of environmental interaction i.e. are highly heritable. The present study investigates the genetic diversity among the cultivated groundnut genotypes and finds association of ISSR markers with resistance against rust and LLS, the two important foliar diseases of this crop worldwide.

Materials and Methods

A total of 20 genotypes of *A. hypogaea* L. were analyzed (Table 1) by using 21 pre-screened ISSR primers (Table 2). These genotypes were grown in experimental fields at Bhabha Atomic Research Centre, Trombay, Mumbai, India in two rainy seasons (June–September) and were evaluated for disease reaction against rust and LLS based on modified 1-9 scale as described in Subhramanyam *et al.* [10]. Young leaves from plants were collected and DNA was extracted using the CTAB procedure with minor modification [11]. DNA concentrations and their purity were determined by taking A_{260} and A_{260}/A_{280} values in V-530 UV-VIS Spectrophotometer (Jasco, Japan). Polymerase chain reaction (PCR) was performed in Mastercycler gradient (Eppendorf, Germany). Reaction mixture for PCR (25 µl) consisted of 1X *Taq* assay buffer (Bangalore Genei Pvt. Ltd., Bangalore, India), 200 µM of each dNTP (Promega, Madison, USA), 0.2 µM of primer (University of British Columbia, Vancouver, Canada), 10 ηg genomic DNA and 1U *Taq* polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). The PCR protocol was as follows: initial denaturation at 94°C for 7 min, 40 cycles' 94°C/30s, 50°C/45s, 72°C/60s and final extension at 72°C/10min. Amplification product were checked on 1.25% agarose gels in 0.5 X TBE buffer and visualized by staining with 0.01% ethidium bromide. For comparison of molecular size of amplicons 100 bp ladder (Bangalore Genei Pvt. Ltd., Bangalore, India) were also run along with each genotype. Only reproducible and distinct bands were scored as 1/0 (presence /absence) for data analysis. Genetic distances were calculated using simple Euclidean

Table 1. List of groundnut genotypes and their field reaction to rust and late leaf spot diseases

S. No.	Genotype	Pedigree	Rust		LLS	
			2004	2005	2004	2005
1	VG 9514	*Arachis cardenasii* X CO 1	1	1	1	1
2	GPBD 4	KRG 1 X ICGV 86855	3	2	2	2
3	GBFDS 272	Not known	1	1	1	1
4	NCAc 343	NC Bunch X PI 1216067	2	2	3	2
5	Mutant 28-2	EMS mutant of VL 1	3	3	2	2
6	DTG 57	TAG 24 X GPBD 4	3	2	1	1
7	DTG 60	TG 26 X Mutant 28-2	1	1	1	1
8	DTG 58	TG 26 X Mutant 28-2	1	1	1	1
9	DTG 27	TG 49 X B 37c	1	1	4	3
10	TDG 56	GPBD 4 X TG 49	3	2	1	1
11	TFDRG 5	TAG 24 X VG 9514	1	1	6	5
12	TMV 2	Mass selection from Gudhiatham bunch	8	9	8	9
13	SB XI	Selection from EC 94943	8	8	9	9
14	JL 24	Ah 4213 X Ah 4354	9	9	9	9
15	TAG 24	TGS 2 X TGE 1	8	9	8	9
16	TG 37A	TG 25 X TG 26	9	9	9	9
17	TG 39	TAG 24 X TG 19	8	7	9	9
18	TG 40	TAG 24 X TG 19	8	6	9	8
19	TPG 41	TG 28A X TG 22	8	9	8	8
20	TG 42	TG 19 X TG 26	8	9	8	9

Note: 1 = no disease and 9 = highly susceptible based on a 1-9 scale of field resistance[10]

distance as $d = \Sigma(x-y)^{1/2}$. The distance matrix was used to construct dendrogram based on Ward method [12] of clustering using STATISTICA software [13]. Polymorphism information content (PIC) for each polymorphic band of a primer was calculated as $1-p^2-q^2$, where, p is band frequency and q is no band (absence of band) frequency [14]. The average PIC for a polymorphic primer was the sum of PIC of all polymorphic bands divided by the number of polymorphic bands. Resolving power for each primer was calculated as $Rp = \Sigma I_b$. where, $I_b = 1-(2 \times |0.5-p|)$, where p is the proportion genotype containing the band [15]. Single marker analysis was used to detect potential association between marker classes (presence or absence of the band) and their respective phenotypic values (disease score). The data on each marker were subjected to the non-parametric Kruskal-Wallis one-way analysis of variance to identify marker potentiality linked to disease [16]. The non-parametric method was chosen instead of parametric ANOVA in view of the ordinal nature of the disease phenotyping data (1-9 scale). The test statistic H that has approximately a chi-square distribution on K-1 (here K = 2) degrees of freedom was tested for its significance and to detect the P value.

Result and discussion

Based on initial screening between resistant genotype, VG 9514 and susceptible genotype, TAG 24, 21 ISSR primers were selected for the present study. Incidentally, all these 21 are dinucleotide repeats with 3' anchored

Table 2. Molecular polymorphism, PIC values and Rp values of ISSR primers among 20 groundnut genotypes

Sr. No.	Primer	Sequence (5'-3')	Total bands	Polymorphic bands	% Polymorphism	PIC	Rp
1	UBC807	$(AG)_8T$	11	8	72.7	0.16	2.2
2	UBC808	$(AG)_8C$	8	6	75.0	0.12	1.1
3	UBC809	$(AG)_8G$	9	9	100.0	0.30	3.6
4	UBC810	$(GA)_8T$	14	13	92.8	0.23	5.0
5	UBC811	$(GA)_8C$	3	2	66.6	0.21	0.9
6	UBC814	$(CT)_8A$	3	3	100.0	0.42	2.0
7	UBC816	$(CA)_8T$	2	2	100.0	0.35	1.0
8	UBC817	$(CA)_8T$	15	8	53.0	0.10	2.0
9	UBC822	$(TC)_8A$	6	1	16.6	0.02	0.1
10	UBC824	$(TC)_8G$	2	2	100.0	0.35	0.9
11	UBC827	$(AC)_8G$	6	4	66.6	0.08	0.5
12	UBC829	$(TG)_8C$	2	2	100.0	0.46	1.5
13	UBC830	$(TG)_8G$	12	9	75.0	0.22	4.0
14	UBC834	$(AG)_8YT$	8	4	50.0	0.11	1.1
15	UBC835	$(AG)_8YT$	11	10	90.9	0.31	4.9
16	UBC836	$(AG)_8YA$	12	9	75.0	0.20	3.8
17	UBC840	$(GA)_8YT$	11	10	90.0	0.10	1.3
18	UBC841	$(GA)_8YC$	2	1	50.0	0.19	0.5
19	UBC842	$(GA)_8YG$	4	2	50.0	0.05	0.2
20	UBC844	$(CT)_8RC$	3	2	66.0	0.12	0.4
21	UBC848	$(CA)_8RC$	10	8	80.0	0.19	2.3
Total =			154	115			
Mean =			7.3	5.47	74.67		

Note: R = (A, G) and Y = (C, T)

either by one or two bases. The PCR amplification using these primers in 20 groundnut genotypes yielded 154 reproducible amplified bands (Table 2). The number of amplified bands varied from 2 (UBC 841) to 15 (UBC 817), and sizes ranged from 200 to 2000 bp. Out of 154 bands, 115 were polymorphic (74.67%). The percentage of polymorphism ranged from 50% (UBC 841, UBC 842) to 100% (UBC 809, 814, 816, 824, 829 and 834) among the primers. Average number of bands and polymorphic bands per primer were 7.3 and 5.5, respectively. A similar type of outcome with only 54.4% polymorphism has been reported employing ISSR technique in analysis of genetic diversity, varietal identification and phylogenetic relationship in groundnut cultivars and its wild species [17]. In mungbean (*Vigna radiata* L. Wilczek), 65.9% polymorphism was revealed and it was higher (83%) when whole set of germplasm comprising varieties, land races and wild accessions was considered [18]. The 3'anchored primers based on 'AG' and 'GA' motifs produced higher polymorphism of 77.27% and 74.85%, respectively. Additionally the primers that were based on 'AG' motifs produced more polymorphic bands on an average (9.83) than the primers based on 'GA' motifs (5.75). Out of 21 ISSR primers used in this study six having 3' anchored 'AG' repeats generated 59 bands (38.3% of total bands) where as four having 3' 'GA' repeats generated 23 bands (14.93 %) (Table 2). Thus the results highlighted that frequency of 'AG'/'GA' repeats is quite higher in groundnut genome [19]. In blackgram (*Vigna mungo* L. Hepper) poly 'GA' and poly 'AG' primers also produce more number of amplified bands [20]. Genetic distances (Euclidean distance) among all possible pairs of cultivated groundnut genotypes ranged from 3.7 (corresponds to 'TG 40 x TPG 41' pair) to 6.9 (corresponds to 'TFDRG 5 x SB XI' pair). Clustering pattern with ISSR data (Fig. 1) revealed two main clusters ('A' and 'B') at a genetic distance of 8.5. The cluster 'A' was further subdivided into two clusters namely 'C' and 'D' at a genetic distance of 6.75. The cluster 'C' grouped four genotypes (VG 9514, DTG 27, TDFRG 5 and GBFDS 272), which were all resistant to rust and LLS. Among them VG 9514 was derived from CO 1 and *Arachis cardenasii* Krapov. et. W. C. Gregory [21], whereas TFDRG 5 was a recombinant line from the cross involving VG 9514 and TAG 24 [22]. The subcluster 'D' grouped five genotypes, which were susceptible to rust, and LLS except NC Ac 343. Whereas cluster 'B' was divided into two subclusters ('E' and 'F') at a genetic distance of 8.1. The subcluster 'E' contained both resistant genotypes (GPBD 4, DTG 57, Mutant 28-2, DTG 60, DTG 58) and susceptible (JL 24 and TMV 2) genotypes. Incidentally DTG 58 and DTG 60 have Mutant 28-2 as donor parent for LLS and rust resistance in their pedigree. Among these, subcluster 'F' contained four genotypes (SB XI, TAG 24, TG 37A and TG 42), which were all susceptible to rust and LLS. Thus, the cluster analysis showed good agreement between molecular data and morphological data in respect of disease reaction and pedigree. To find out any relation between the marker data and disease reaction, single ANOVA was made based on Kruskal-Wallis analysis. The two polymorphic markers namely UBC 810_{540} (Hc = 5.64, $0.05<P< 0.01$) and UBC 810_{500} (Hc = 5.92, $0.05<P< 0.01$) were found significantly associated with LLS resistance. Whereas UBC 810_{540} (Hc = 5.12, $0.05<P< 0.01$) associated with only rust resistance. The primer UBC 810 contained 'GA' repeats with 3' anchored single base T and its two polymorphic bands were found associated with resistance to rust and LLS. Recently, three 'GA' based simple sequence repeat markers

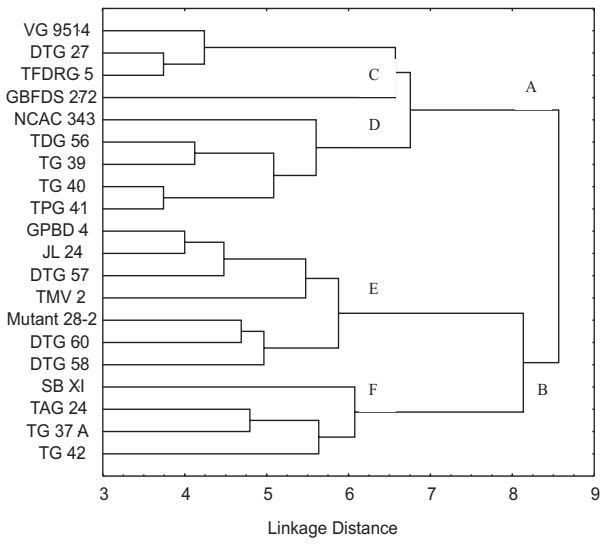

Fig. 1. Dendrogram showing relationship among groundnut genotypes based on ISSR profile

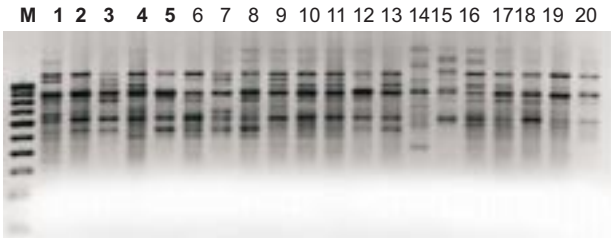

Fig. 2. Amplification profile of 20 groundnut genotypes employing primer UBC 810. Note: M=100 bp Ladder, Lane 1-20 were numbered as per the genotypes name in Table 1

(pPGPseq-17F6, pPGPseq-16C6 and pPGPseq-10D4) were found associated with rust and LLS resistance based on AMOVA and Kruskal-Wallis one-way ANOVA [16]. The PIC as a relative measure of informativeness, ranged between 0.10 (UBC 840) to 0.31 (UBC 835) (Table 2). The estimation of Rp value revealed a large variation (2.0 to 18) among primers (Table 2). The highest Rp value was obtained in primer UBC 810 (Fig. 2) and UBC 835. Since, the higher PIC and Rp values indicate more informativeness for genotype discrimination and diversity studies, these primers would be preferred for use in subsequent fingerprinting research in cultivated groundnut. A similar type of findings was obtained in fingerprinting of groundnut accessions where PIC content varied from 0.1 to 0.5 and Primer Index varied from 0.35 to 1.73 for ISSR primers [17]. These findings among different cultivated groundnut genotypes support utility of ISSR primer in genetic diversity studies. The present work indicates high level of genetic variation among cultivated groundnut genotypes, which otherwise show a low level of polymorphism in different studies [5, 6, 7, 8]. Therefore ISSR markers serve as a basis for future work on DNA fingerprinting of closely related germplasm, tagging of agronomic traits and linkage mapping in cultivated groundnut.

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References

1. **Krapovickas A. and Gregory W. C.** 1994. Taxonomy of the genus *Arachis* (Leguminosae). Bonplandia, **8**: 1-186.
2. **Subrahmanyam P., Rao V. R., McDonald D., Moss J. P. and Gibbons R. W.** 1989. Origins of resistance to rust and late leaf spot in peanut (*Arachis hypogaea* Fabaceae). Econ. Bot., **43**: 444-455.
3. **Dwivedi S. L., Crouch J. H., Nigam S. N., Ferguson M. E. and Paterson A. H.** 2003. Molecular breeding of groundnut for enhanced productivity and food security in the semi-arid tropics, opportunities and challenges. Adv. in Agronomy, **80**: 153-221.
4. **Bianchi-Hall C., Keys R. and Stalker H.** 2000. Use of protein profiles to characterize peanut cultivars. News Assoc. official seed anal., **65**: 25-26.
5. **Halward T. M., Stalker H. T., Larue E. A. and Kochert G.** 1991. Genetic variation detectable with molecular markers among unadapted germplasm resources of cultivated peanut and related wild species. Genome, **34**: 1013-1020.
6. **Subramanian V., Gurtu S., Rao R. C. N. and Nigam S. N.** 2000. Identification of DNA polymorphism in cultivated groundnut using random amplified polymorphic DNA (RAPD) assay. Genome, **43**: 656-660.
7. **Mondal S., Ghosh S. and Badigannavar A. M.** 2005. RAPD polymorphism among groundnut genotypes differing in disease reaction to late leaf spot and rust. Int. Arachis Newslett., **25**: 27-30.
8. **Herselman L.** 2003 Genetic variation among southern African cultivated peanut (*Arachis hypogaea* L.) genotypes as revealed by AFLP analysis. Euphytica, **133**: 319-327.
9. **Zietkiewiczk E., Rafalski A. and Labuda D.** 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics, **20**: 176-183.
10. **Subrahmanyam S., McDonald D., Waliyar F., Reddy L. J., Nigam S. N., Ramanatha Rao V., Singh A. K., Pande S., Reddy P. M. and Subha Rao P. V.** 1995. Screening methods and sources of resistance to rust and late leaf spot of groundnut. Information Bulletin, No. 47. International Crops Research Institute for the Semi-Arid Tropics, Patnacheru 502324, Andhra Pradesh, India.
11. **Saghai Maroof M. A., Saliman K. M., Jorgensen R. A. and Allard R. W.** 1984. Ribosomal DNA spacer length polymorphism in barley, Mendelian Inheritance chromosomal location and population dynamics. Proc. Natl. Acad. Sci. USA, **81**: 8014-8018.
12. **Ward J. H.** 1963. Hierarchical grouping to optimize an objective function. J. of the Am. Statistical Assoc., **58**: 236.
13. **Statistica.** 1996. STATISTICA version 6.0, Statsoft Inc., Tulsa, USA.
14. **Ghislain M., Zhang D., Fajardo D., Huaman Z. and Hijmans R. J.** 1999. Marker-assisted sampling of the cultivated Andean potato Solanum phureja collection using RAPD markers. Genet. Resources and Crop Evol., **46**: 547-555.
15. **Prevost A. and Wilkinson M. J.** 1999. A new system of comparing primers applied to ISSR fingerprinting of potato cultivars. Theor. Appl. Genet., **98**: 107-112.
16. **Mace E.S., Phong D. T., Upadhyaya 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**: 317-330.

17. **Raina S. N., Rani V., Kojima T., Ogihara Y., Singh K. P. and Devarumath R. M.** RAPD and ISSR fingerprint as useful genetic markers for analysis of genetic diversity, varietal identification and phylogenetic relationship in peanut (*Arachis hypogaea* L.) cultivars and wild species. Genome, **44**: 763-772.

18. **Chattopadhyay K., Nasim A. M., Sarkar H. K., Mandal N. and Bhattacharya S.** 2005. Diversity analysis by RAPD and ISSR markers among the selected mungbean [*Vigna radiata* (L.) Wilczec] genotypes. Indian J. Genet., **65**: 173-175.

19. **Ferguson M.E., Burrow M. D., Schulze S. R., Bramel P. J., Paterson A. H., Kresovich S. and Mitchell S.** 2004 Microsatellite identification and characterization in peanut (*Arachis hypogaea* L.). Theor. Appl. Genet., **108**: 1064-1070.

20. **Souframanien J. and Gopalkrishna T.** 2004. A comparative analysis of genetic diversity in blackgram genotypes using RAPD and ISSR markers. Theor. Appl. Genet., **109**: 1687-1693.

21. **Varman P. V.** 1999. A foliar disease resistant line developed through interspecific hybridization in groundnut (*Arachis hypogaea* L.). Indian J. Agric. Sci., **69**: 67-68.

22. **Badigannavar A. M., Kale D. M., Mondal S. and Murty G. S. S.** 2005. Trombay groundnut recombinants resistant to foliar diseases. Mutation Breeding Newsl. and Rev., **1**: 11-12.