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Assessment of genetic diversity among selected groundnut germplasm. I: RAPD analysis

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Abstract

Assessment of genetic diversity in a crop species is prerequisite to its improvement. The use of germplasm with distinct DNA profiles will help to generate genetically diversified breeding populations. The aims of the present experiment were to study molecular diversity among selected groundnut accessions and identify those with distinct DNA profiles for mapping and genetic enhancement. Twenty-six accessions and eight primers of a 10-mer were selected for random amplified polymorphic DNA assay. The genetic similarity (S_{ij}) ranged from 59.0% to 98.8%, with an average of 86.2%. Both multidimensional scaling and unweighted pair-group method with arithmetic averages (UPGMA) dendrograms revealed the existence of five distinct clusters. However, this classification could not be related to known biological information about the accessions falling into different clusters. Some accessions with diverse DNA profiles (ICG 1448, 7101, and 1471, and ICGV 99006 and 99014) were identified for mapping and genetic enhancement in groundnut.

Key words: *Arachis hypogaea* — aflatoxin — genetic diversity — interspecific derivatives — RAPD

Cultivated groundnut (*Arachis hypogea* L.) is a highly self-pollinated, allotetraploid annual legume with $2n = 40$. It is classified based on the presence or absence of flowers on the main axis into two subspecies, *hypogaea* (Krap. *et* Rig.) and *fastigiata* (Waldron). Subspecies *fastigiata*, in turn, is classified into four botanical varieties: *fastigiata*, *peruviana* Krapov. & W.C. Gregory, *aequatoriana* Krapov. & W.C. Gregory and *vulgaris* C. Hartz. The two botanical varieties in the subspecies *hypogaea* are *hypogaea* and *hirsuta* Kohler. These botanical varieties have distinguished morphological characteristics (Singh and Nigam 1997). Despite significant morphological variation in groundnut, the lack of variability at the genetic level is often cited as one of the reasons for little progress in genetic enhancement of the crop (Norden et al. 1982).

Knowledge of genetic diversity in a crop species is fundamental to its improvement. A variety of molecular, chemical, and morphological descriptors are used to characterize the genetic diversity among and within crop species. Molecular marker techniques including random amplified polymorphic DNA (RAPD) (Williams et al. 1990) have been used to study polymorphism in groundnut. Very low or no polymorphism in cultivated types to abundant polymorphism in wild *Arachis* has been reported (Halward et al. 1991, 1992, Lanham et al. 1992, Paik-Ro et al. 1992). However, recent studies revealed polymorphism in the cultivated groundnut using DNA amplification fingerprinting (DAF) and amplified fragment length polymorphism (AFLP) (He and Prakash 1997), RAPD (Bhagwat et al. 1997, Subrahmanian et al. 2000), and simple sequence repeat (SSR) (Hopkins et al. 1999) assays. The low level of polymorphic variation in cultivated groundnut is attributed to its origin from a single polyploidization event that occurred relatively recently on an evolutionary time scale (Young et al. 1996). Limited germplasm, evaluated in these studies could also explain the small polymorphic variability observed in cultivated groundnut. Identification of sufficient polymorphic variation among germplasm is necessary to facilitate marker-assisted breeding.

The aims of the present study were: (1) to evaluate molecular diversity among cultivated groundnut germplasm, cultivars, and interspecific derivatives; and (2) to identify germplasm with distinct DNA profiles for mapping and genetic enhancement in groundnut.

Materials and Methods

Plant materials: Twenty-six accessions of groundnut, *Arachis hypogaea* L., consisting of interspecific derivatives, land races and released cultivars were selected to study molecular diversity following RAPD assay. Their origin, pedigree, and other characteristics are described in Table 1. Although groundnut is highly self-pollinated, occasional outcrossing due to bees can introduce variability into an accession. These accessions were grown for two seasons under greenhouse conditions prior to study to remove any within-accession phenotypic diversity. Ten seeds from each accession were sown in pots in a greenhouse. Young leaves from 2-week-old plants were bulk harvested for each accession and immediately placed in liquid nitrogen for DNA extraction.

DNA extraction: DNA was extracted based on a previously reported cetyltrimethylammonium bromide (CTAB) method (Saghai-Maroof et al. 1984) with some modification. Leaves were ground to fine powder in the presence of liquid nitrogen and transferred to a sterile tube containing 9 ml of preheated (65°C) 2 × CTAB extraction buffer (100 mM Tris-HCl buffer pH 8, 700 mM NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA) pH 8, 2% hexadecyltrimethyl-ammonium bromide, 1% β-mercaptoethanol and 1% sodium bisulphite). 200 mg polyvinylpyrrolidone 10 per g of leaf tissue was added and mixed gently. The contents were incubated for 90 min at 65°C in a water bath with occasional shaking during incubation. The tubes were kept for 10 min to allow them to return to room temperature. An equal quantity (9 ml) of chloroform and amyl alcohol solution, prepared in a ratio of 24 : 1, was added to the tubes and they were

Table 1: Origin, pedigrees and botanical characteristics of groundnut (*Arachis*) accessions used for molecular diversity analysis by random amplified polymorphic DNA analysis

Accession[1]	Pedigree	Origin	Biological status[2]	Botanical variety	Seeds per pod	Seed colour[3]	Reaction to abiotic and biotic stresses
ICGV 99001	'Robut 33-1' × *A. villosa*	India	ID	*vulgaris*	2-1-3	DR	Resistant to rust and late leaf spot
ICGV 99002	'Chico' × ('Shulamit' × *A. correntina* × *A. batizocoii*)	India	ID	*hypogaea*	2-3-1	DR	Resistant to rust
ICGV 99003	*A. hypogaea* × (*A. cardenasii* × *A. stenosperma*)	India	ID	*hypogaea*	2-1-3	DR	Resistant to rust
ICGV 99004	TMV 2 × (*A. hypogaea* × *A. cardenasii*)	India	ID	*vulgaris*	2-1	DR	Resistant to rust and late leaf spot
ICGV 99005	TMV 2 × (*A. hypogaea* × *A. batizocoii* × *A. duranensis*)	India	ID	*hypogaea*	2-1-3	DR	Resistant to rust
ICGV 99006	*A. hypogaea* × *A. cardenasii*	India	ID	*vulgaris*	2-1-3	DR	Resistant to rust and late leaf spot
ICGV 99007	*A. hypogaea* × *A. cardenasii*	India	ID	*hypogaea*	2-1	DR	Resistant to rust
ICGV 99008	'Robut 33-1' × *A. villosa*	India	ID	*hypogaea*	2-1-3	DR	Resistant to rust
ICGV 99009	*A. hypogaea* × *A. cardenasii* × ICG 4751	India	ID	*hypogaea*	2-3-1	DR	Resistant to rust
ICGV 99010	*A. hypogaea* × *A. cardenasii* × ICG 4751	India	ID	*hypogaea*	2-1	DR	Resistant to late leaf spot
ICGV 99011	ICGMS 42 × (*A. hypogaea* × *A. cardenasii*)	India	ID	*hypogaea*	2-3-1	T	Resistant to late leaf spot
ICGV 99012	ICGMS 42 × (*A. hypogaea* × *A. cardenasii*)	India	ID	*hypogaea*	2-3-1	T	Resistant to rust
ICGV 99013	*A. hypogaea* × *A. cardenasii*	India	ID	*vulgaris*	2-1-3	DR	Resistant to rust and late leaf spot
ICGV 99014	*A. hypogaea* × *A. cardenasii*	India	ID	*vulgaris*	2-1	T	Resistant to rust and late leaf spot
ICGV 99015	TMV 2 × (*A. hypogaea* × *A. batizocoii* × *A. duranensis*)	India	ID	*hypogaea*	2-1-3	DR	Resistant to rust
ICGV 99016	TMV 2 × (*A. hypogaea* × *A. batizocoii* × *A. duranensis*)	India	ID	*hypogaea*	2-3-1	DR	Resistant to rust
ICG 10021		Peru	LR	*peruviana*	3-2-1	DP	Resistant to rust and cold tolerant
ICG 4751		USA	–	*vulgaris*	2-1	T	
ICG 1448		USA	–	*vulgaris*	2-1	T	Resistant to aflatoxin
ICG 7101		India	LR	*vulgaris*	2-1	T	Resistant to aflatoxin
ICG 4749		Argentina	LR	*vulgaris*	2-1	T	Resistant to seed colonization
ICG 1326	AH 4218 × AH 4354	India	RC	*vulgaris*	2-1	T	Resistant to seed colonization
ICG 1471	Selection from exotic germplasm from Argentina	Senegal	RC	*vulgaris*	2-1	T	Resistant to seed colonization
ICG 799	Selection from 'Robut'	India	RC	*hypogaea*	2-1	T	Tolerant to peanut bud necrosis disease
ICG 3042	Selection from 'Kadiri 2'	Nigeria	RC	*hypogaea*	2-1	T	
ICG 5285		Israel	RC	*hypogaea*	2-1	R	

[1] ICG, ICRISAT groundnut; ICGV, ICRISAT groundnut variety.
[2] LR, land race; RC, released cultivar; ID, interspecific derivatives.
[3] DR, dark red; T, tan; DP, dark purple; R, rose.

Table 2: Oligonucleotide primer with number of amplified fragments, polymorphic fragments and polymorphism (%) in groundnut

Primer	Amplified fragments (N_A)	Polymorphic fragments (N_P)	Polymorphism ($P = N_P/N_A \times 100$)	Fragments per primer
A9	123	29	23.58	4.73
A19	101	19	18.81	3.88
B11	83	26	31.32	3.19
G10	149	18	12.08	5.73
GN20	126	11	8.73	4.85
GN26	143	18	12.58	5.50
H4	84	12	14.28	3.23
N4	130	43	33.08	5.00
Total	939	176	18.74 (average)	4.51 (average)

rotated on a tube rotator for 10 min and centrifuged at 5000 r.p.m. at 15°C for 20 min. The aqueous phase was transferred to a clean tube, and the chloroform and amyl alcohol solution step was repeated. Nucleic acids were precipitated by adding 0.6 ml chilled isopropanol to the aqueous phase and incubating at −20°C for 20 min. The DNA was spooled using glass Pasteur pipettes and transferred to a new sterile tube containing 2 ml of $T_{50}E_{10}$ buffer (50 ml $T_{50}E_{10}$ + 1 ml RNase 10 mg/ml) and left overnight at room temperature. Contents were later on incubated at 37°C for 30 min. 150 μl of 5 M NaCl was added to the tube which was kept at 4°C. An equal volume (150 μl) of solution of phenol, chloroform, and amyl alcohol, prepared in a ratio of 25 : 24 : 1, was added to the tube and mixed gently and the tube was centrifuged at 5000 r.p.m. at 2°C. The clear phase was once again cleaned using another phenol, chloroform and amyl alcohol solution, then washed and spun at 2°C. The aqueous phase was transferred to new tubes and DNA was precipitated using 2–4 ml of pure chilled ethanol. Tubes were kept at −20°C for 10 min. The DNA precipitate was removed and washed with 2 ml of 0.2 m sodium acetate in 76% alcohol for 20 min followed by 1 ml of 10 mM ammonium acetate in 76% alcohol for 1 min. The DNA pellet was further washed with 70% alcohol for 30 min and centrifuged again. The tubes were allowed to drain and dried at room temperature for 2–3 h, then resuspended in 200–500 μl of Tris EDTA buffer. The quality and concentration of DNA was assessed by a spectrophotometer and also by gel electrophoresis using 0.8% agarose with known concentrations of uncut lambda DNA.

RAPD amplification: Eight oligonucleotide primers (A9, A19, B11, G10, GN20, GN26, H4 and N4) of 10-mer, each with at least 60% G + C content, were obtained from Operon Technologies, Inc., Alameda, CA. Polymerase chain reactions (PCRs) were performed following Williams et al. (1990) with a modified mixture. The reaction mixture (20 μl) contained 10 × PCR buffer (10 mM Tris-HCl, pH 8.3), 50 mM KCl, 0.01% gelatin, 25 mM $MgCl_2$, 100 mM each of dATP, dCTP, dGTP and dTTP, 20 ng primer, 25 ng of genomic DNA and 1 unit of *Taq* DNA polymerase (Promega, Madison, WI). Amplification was performed in 0.2 ml thin-walled tubes placed in a thermal cycler (9600, Perkin Elmer, Norwalk, CI). The samples were subjected to 45 repeats of the following cycle: 92°C 1 min, 35°C for 1 min, 72°C for 2 min. The amplified products were analysed by electrophoresis in 1.4% gels stained in ethidium bromide (10 mg/ml) and run in 1 × Tris borate buffer EDTA buffer at 70 V for 2 h. The gels were photographed under UV light with Polaroid film. The amplified fragments were scored as '1' for the presence and '0' for the absence of a band from higher to lower molecular weight products.

Statistical analysis: Pair-wise genetic similarities (S_{ij}) between accessions i and j were estimated using the similarity coefficient of Nei and Li (1979) as $S_{ij} = 2\,N_{ij}/(N_i + N_j)$, where N_{ij} is the number of bands in common in accessions i and j, and N_i and N_j are the total number of bands in accessions i and j, respectively. S_{ij} represents the proportion of bands in common between any two accessions and may range from 0 (no common bands) to 1 (identical band profile for the two accessions). Based on genetic distance estimates, $D_{ij} = 1 - S_{ij}$, multidimensional scaling (MDS) (Kruskal and Wish 1978) was performed to see whether the observed molecular variation indicated any evidence of clustering among accessions. A dendogram was constructed based on the S_{ij} values by adopting the sequential hierarchical agglomerative non-overlapping (SHAN) clustering technique of unweighted pair group method of arithmetic means (UPGMA), which is a variant of the average linkage clustering algorithm (Sneath and Sokal 1973). The dendrogram was truncated at a similarity threshold value at which well-separated clusters, as indicated by MDS, were obtained. All computations were performed using the statistical computing package Genstat5 Release 4.1.

Results

Eight primer data on 26 accessions generated 939 amplified fragments, and 176 (18.74%) were polymorphic (Table 2). The polymorphism among primers ranged from 8.73%, with primer GN20 to 33.08% with primer N4. The amplified fragments per primer ranged from 3.19 with primer B11 to 5.73 with primer G10, with an average of 4.51 per primer. DNA polymorphism detected with primer H4 on 17 accessions is shown in Fig. 1.

The S_{ij} values for 325 pairwise comparisons among 26 accessions ranged from 59.0% to 98.8%, with an average of 86.2%. Most accessions, including interspecific derivatives, showed high degrees of genetic similarity. However, ICGV 99006 with ICGV 99014 (75.4%) and ICG 1448 with ICG 7101 (62.1%), ICG 1471 (64.3%), ICG 4749 (65.3%), ICG 1326 (65.5%), ICGV 99005 (59%) and ICGV 99012 (59.0%) showed low S_{ij} values.

The MDS plot based on the first two coordinates and the corresponding UPGMA dendogram are presented in Fig. 2. There is clear evidence of two well-separated clusters in the MDS plot that corresponds to a similarity threshold of 60% in the dendogram. The larger cluster can be further

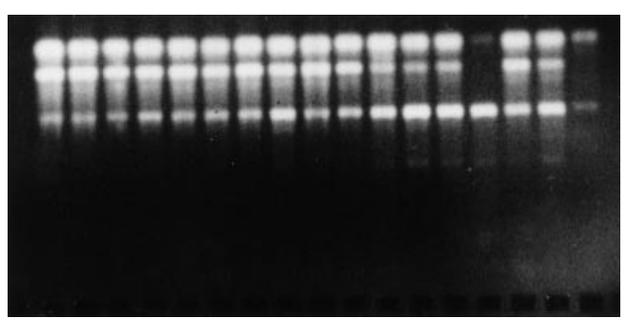

Fig. 1: Random amplified polymorphic DNA pattern of variation in 17 groundnut accessions generated by primer H4

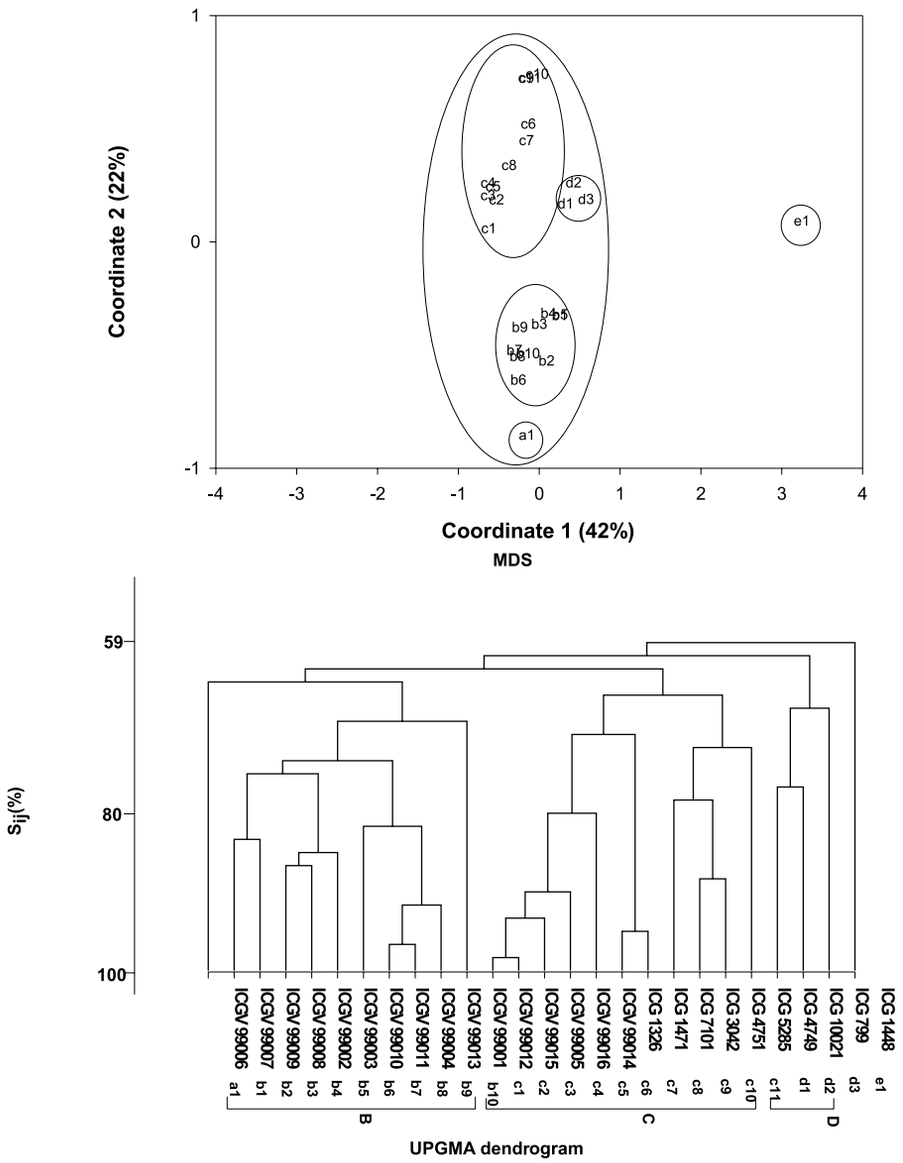

Fig. 2: Multidimensional scaling (MDS) and unweighted pair-group method with arithmetic averages (UPGMA) dendrogram of groundnut accessions generated from random amplified polymorphic DNA data. The scale for the dendrogram is based on the Nei and Li (1979) similarity coefficient (S_{ij})

partitioned into four subclusters. These five clusters in the MDS plot correspond to five clusters in the dendogram truncated at a 66% similarity threshold. ICG 1448 and ICGV 99006 maintained their separate identity both in MDS and UPGMA dendrograms; other interspecific derivatives grouped in either cluster B or cluster C. Cluster C also contained accessions that are highly susceptible to rust, *Puccinia arachidis* Spegazzini, and late leaf spot (LLS), *Phaeoisariopsis personata* Berk. & Curtis van Arx. ICG 4751, the crinkle leaf mutant, grouped with other accessions that have normal leaves in cluster C. ICG 10021, a Peruvian landrace that belongs to the subspecies *fastigiata* var. *peruviana* and possesses resistance to rust and tolerance to cold temperatures, did not cluster with rust and/or LLS resistant interspecific derivatives.

Discussion

The pair-wise estimates of genetic similarities (S_{ij}) revealed that most of the accessions that included interspecific derivatives showed a high degree of genetic similarity at the molecular level following RAPD assay. Although the cluster analysis grouped accessions into five different clusters, the accessions in each cluster could not be associated with their country of origin, known parentage (pedigree), subspecies classification, pod and seed characteristics, and their reaction to abiotic and biotic stresses.

Wild *Arachis* species such as *A. villosa*, *A. correntina*, *A. batizocoi*, *A. cardenasii*, *A. stenosperma* and *A. duranensis* had revealed abundant polymorphism in previous studies (Paik-Ro et al. 1992, Halward et al. 1991, 1992). However, the interspecific derivatives involving these species developed at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India, and used in the present study showed a high degree of genetic similarity with cultivated groundnut germplasm. These interspecific derivatives are cytologically stable tetraploids and it is possible that much of the molecular variation originating from *A. hypogaea* × wild *Arachis* species crosses was lost in early generations because of the selection for phenotypic traits

closely resembling the cultivated groundnut. Crosses involving wild species should, therefore, be assessed both for phenotypic and molecular variation to retain a high proportion of the wild *Arachis* genome associated with useful phenotypic traits for further genetic enhancement by marker-assisted selection in groundnut.

A few accessions in the present study showed low degrees of genetic similarity in their DNA profiles. These were ICGV 99006 with ICGV 99014 and ICG 1448 with ICG 7101, 1471, 4749, and 1326, and ICGV 99005 and 99012. Although ICGV 99006 and 99014 have common parentage, belong to the subspecies *fastigiata* var. *vulgaris* and possess resistance to both rust and LLS, they differ in seed colour. The former has dark-red seeds while the latter has tan coloured seeds. In addition, there may be differences for other morpho-physiological and reproductive traits between ICGV 99006 and ICGV 99014. Despite the close similarity for botanical types (subspecies *fastigiata* var. *vulgaris*) and agronomic traits between the two accessions (ICG 1448 and ICG 7101) resistant to aflatoxin production, they revealed considerable variation in their DNA profiles. There is a possibility that they may possess different genes for these traits. The low genetic similarity of ICG 1448 with ICG 1471, 4749 and 1326 (all belong to the subspecies *fastigiata* var. *vulgaris* and have similar phenotypic traits) is probably associated with differences in their seed colonization and aflatoxin production by *Aspergillus flavus*. ICG 1448 is resistant to aflatoxin production but is susceptible to seed colonization. Conversely, ICG 1471, 4749 and 1326 are resistant to seed colonization but susceptible to aflatoxin production (Mehan et al. 1986, 1987, Naguib et al. 1989). The low genetic similarity of ICG 1448 with ICGV 99005 and 99012 is probably associated with their differences in botanical type and reaction to rust disease. The first belongs to the subspecies *fastigiata* var. *vulgaris*, and is susceptible to rust, whereas last two belong to the subspecies *hypogaea* var. *hypogaea* and are resistant to rust.

The estimates of genetic relationships can be useful for organizing germplasm for conservation of genetic resources, for the identification of cultivars, for selection of parents for hybridization, for predicting favourable heterotic combinations and for reducing the number of accessions needed to ensure sampling a broad range of genetic variability in breeding programmes. Accessions with the most distinct DNA profiles are likely to contain the greatest number of novel alleles. It is in these accessions that one is likely to uncover the largest number of unique and potentially agronomically useful alleles. This strategy has resulted in a high proportion ($\approx 50\%$) of new and useful quantitative trait loci (QTL) alleles in rice and tomato (Tanksley and McCouch 1997). ICG 1471 has been crossed with ICG 1448 and ICG 7101 to develop recombinant inbred lines for identifying DNA markers and QTL linked with resistance to seed colonization and aflatoxin production by *A. flavus*. ICGV 99006 was crossed with ICGV 99014 to develop populations with enhanced resistance to rust and LLS, and ICG 1448 with ICG 7101 for resistance to aflatoxin production. Further studies are envisaged to quantify the genetic gains in populations derived from accessions with distinct DNA profiles.

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