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# Mutational origin of genetic diversity in groundnut (*Arachis hypogaea* L.)

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**Abstract**

**To investigate the mutational origin and nature of genetic diversity in groundnut, mutants derived from Dharwad Early Runner and others along with natural types, and intra and interspecific derivatives belonging to all the four botanical types were assessed for their morphological, biochemical and molecular diversity. Similarity among the natural and mutant categories suggests the key role of mutations in creating enormous diversity in terms of different subspecies and botanical types of groundnut. Behaviour of the Dharwad Early Runner derived mutants and several unusual features are indicative of the non-Mendelian turnover mechanisms. Multiple gene differences between the mutants and their parents, paternal inheritance and tissue-specific expression of glutamate oxloacetate transaminase isozyme, response of mutants to 5-azacytidine (a demethylating agent) and limited molecular diversity compared to enormous morphological diversity suggests the possible involvement of epigenetic mechanisms in the differentiation of groundnut into different subspecies and botanical varieties.**

**Key words:** Peanut, isozyme, RAPD, 5-azacytidine, epigenetic mechanism, intraspecific differentiation

## Introduction

Groundnut (*Arachis hypogaea* L.) is an important oilseed crop, which is cultivated in more than 96 countries of the world. It is an annual legume and a rich source of edible oil and proteins. About two thirds of the world production is crushed for oil and the remaining one third is consumed as food. Cultivated groundnut has enormous morphological diversity based on which it is classified into ssp. *fastigiata* Waldron and ssp. *hypogaea* Krapov and Rigoni. The ssp. *hypogaea* is characterized by alternate branching, absence of flowers on the main axis, long duration (120-150 days) and presence of seed dormancy, while the ssp. *fastigiata* has characters like sequential flowering, presence of flowers on the main axis, short duration (85-130 days) and lack of seed dormancy. Further, based on the morphological differences, spp. *fastigiata* is divided into four botanical varieties *viz*., *fastigiata*, *peruviana* Krapov. and W. C. Gregory, *aequatoriana* Krapov. and W. C. Gregory, and *vulgaris* C.Harz, while ssp. *hypogaea* into var. *hypogaea* and *hirsuta* [1]. However, Virginia bunch and Virginia runner (var. *hypogaea*), Valencia (var. *fastigiata*) and Spanish (var. *vulgaris*) are the cultivated botanical types.

In groundnut breeding programme at University of Agricultural Sciences, Dharwad, a true breeding variant, 'Dharwad Early Runner' (DER) was isolated from a cross between 'Dh 3-20' and 'CGC1' [2]. It is an intermediate type which shares the features of ssp. *fastigiata* like sequential flowering, presence of flowers on the main axis and short duration and features of ssp. *hypogaea* like prostrate habit with short main axis, small seeds and fresh seed dormancy. DER on treatment with ethyl methane sulphonate (EMS), gamma rays and 5-azacytidine resulted in a very high frequency of mutants belonging to all the four cultivated botanical types [3,4]. Most of the mutants bred true but some of the mutants continued to segregate and produced all other botanical types. Several unusual features were observed like high frequency of mutations and reversions, non-random mutations, segregation distortions, unstable variants in advanced generations, somatic mutations, multiple character mutations, multiple alternate forms, homozygous mutations and mutation outbursts which would be difficult to explain through conventional mutation theory [3].

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Diverse mutants that are derived from the same source (DER) constitute an excellent material for probing the mutational origin and nature of genetic diversity in groundnut. Hence, the mutants and natural types belonging to different botanical types were assessed for their diversity with respect to morphological traits, native proteins, glutamate oxaloacetate transaminase (GOT) isozyme and randomly amplified polymorphic DNA (RAPD) assay to know the mutational origin of diversity in groundnut.

## Materials and methods

### *Plant materials*

In this study, 36 genotypes comprising natural types (10), DER and its mutants (15), other mutants (3), interspecific derivatives (5) and other genotypes (3) belonging to four botanical types were used (Table 1).

### *Morphology*

Morphological characterization of 36 genotypes for 12 morphological characters viz. growth habit, branching pattern, main stem flowering, main stem height (cm), leaflet shape, leaflet length (cm), leaflet width (cm), leaflet tip, pod reticulation, pod constriction, pod beak and pod size was done according to the 'Groundnut Descriptors' [5]. In addition, they were also assessed for productivity parameters like pod weight (g/plant), shelling out-turn (%) and test weight (g) and reaction to late leaf spot and rust diseases.

### *Native proteins*

Native proteins were extracted from hypocotyls of six day-old seedlings using phosphate buffer (0.1 M, pH 8.0) with 1% polyvinyl pyrollidone (PVP). Resolving gel (7.5% acrylamide) was prepared according to the method of Hames and Rickwood [6] and profiling by polyacrylamide gel electrophoresis was done as per the method of Davis [7]. Gel was stained by using 0.1% Coomassie Brilliant Blue R 250 in methanol, acetic acid and water (5:2:5 v/v). Banding pattern of all the genotypes was recorded and each band was given a Relative mobility ($R_m$) value, which is the ratio between the distance traveled by the molecule and distance traveled by the tracking dye (bromophenol blue). Comparison was made between the genotypes for their electrophoretic patterns.

### *Glutamate oxaloacetate transaminase (GOT) isozyme*

GOT isozyme was extracted from hypocotyls and flowers using phosphate buffer (0.1 M, pH 6.8) with 1% PVP. Gel preparation and profiling was done similar to that of native proteins. GOT isozyme-specific staining was done as described in Tanksley and Orton [8] and gels were incubated in dark at 30$^o$ C for 2 to 4 hours or until blue bands appear. GOT isozyme profile of all the genotypes in hypocotyls and flowers was recorded and each band was given the $R_m$ value. Comparison between the genotypes was made for their GOT isozyme profile in hypocotyls and flowers.

### *DNA extraction and RAPD assay*

DNA was extracted from young leaves of 15 day-old seedlings using urea-based plant DNA mini-prep method [9] with some modifications. The quality and concentration of DNA was assessed by using spectrophotometer readings [DNA (μg/ml) = $OD_{260}$ x dilution factor x 50 μg] and also by gel electrophoresis (0.8% agarose) with known concentrations of uncut lambda DNA. Purified genomic DNA extracts (20 ng) were used as template DNA. The PCR amplification [94$^o$ C for 4 min (1 cycle); 94$^o$ C for 1 min, 37$^o$ C for 1 min, 72$^o$ C for 2 min (40 cycles); 72$^o$ C for 10 min (1 cycle); 4$^o$ C storage] using the random primers was done as per Bhagwat *et al*. [10] with some modifications and amplified fragments were separated on 1.2% agarose gel using 1X TAE buffer (pH 8.0) and documented using white/2UV Trans-illuminator of Ultra Violet products, London. Thirty out of thirty-six genotypes were selected for RAPD assay by excluding Kopergaon 3, Mardur Local, B 37c, B 31J, ICGV 86699 and ICGV 86155 without loosing the representation from all types. They were subjected to PCR amplification by using 21 random primers (OPK10, OPK12 to 19, OPF20, OPAT1 to 9, OPA15 and OPA18) from Operon Technologies Inc, Alamedas, USA. The amplification profile of all 30 genotypes for all the primers was scored depending on the presence (1) or absence (0) of amplified fragments and 0 and 1 matrix was constructed.

### *Statistical analysis*

The observations recorded on all the 17 morphological characters were subjected to principal component analysis as described by Dhrymes [11]. The genotypes were grouped into clusters based on the contribution of each character towards total variation using statistical package for agricultural research (SPAR) software. The data on native proteins and RAPD was subjected to diversity analysis using NTSYS-pc 2.0 program. The genetic similarity was computed using DICE coefficient, clustering was done using SAHN and dendrogram was constructed by unweighted paired group method using arithmetic averages (UPGMA). Polymorphism information content (PIC) for each primer in RAPD assay

**Table 1.** List of genotypes used and their pedigree

| S.No. | Class and genotypes | Botanical variety [a] | Pedigree |
|---|---|---|---|
| | **Natural types** | | |
| 1 | PI 393516 | VL | ICG 7888 (Peru) |
| 2 | NCAc 17090 | VL | ICG 1697 (Peru) |
| 3 | Kopergaon 3 | VL | Selection from local |
| 4 | TMV 2 | SB | Mass selection from Gudhiatham bunch |
| 5 | JL 24 | SB | Selection from EC 94943 |
| 6 | KRG 1 | SB | Selection from Argentine |
| 7 | Chitra | VB | Spanish 58-1 x EC 1688 |
| 8 | S 230‘ | VR | Selection from Tandur local |
| 9 | Mardur Local | VR | Selection from local condition |
| 10 | M 13 | VR | Selection from NC 13 |
| | **Induced mutants** | | |
| 11 | NLM | VR | EMS mutant of TMV 2 |
| 12 | Mutant 28-2 | VL | EMS mutant of VL 1 |
| 13 | DER[b] | - | Dh 3-20 x CGC-1 |
| 14 | VL 1 | VL | EMS mutant of DER |
| 15 | VL 2 | VL | EMS mutant of DER |
| 16 | VL 3 | VL | EMS mutant of DER |
| 17 | SB 2 | SB | EMS mutant of DER |
| 18 | SB 3 | SB | Gamma rays mutant of DER |
| 19 | SB 6 | SB | Gamma rays mutant of DER |
| 20 | VB 2 | VB | EMS mutant of DER |
| 21 | VB 8 | VB | EMS mutant of VL 1 |
| 22 | VB 9 | VB | EMS mutant of VL 1 |
| 23 | VR 1 | VR | EMS mutant of DER |
| 24 | VR 5 | VR | 5-azacytidine mutant of DER |
| 25 | VR 8 | VR | EMS mutant of VL 1 |
| 26 | JLVR 1 | VR | Spontaneous mutant of JL 24 |
| 27 | JLVR 2 | VR | Spontaneous mutant of JL 24 |
| | **Interspecific derivatives** | | |
| 28 | GPBD 4 | SB | KRG 1 x CS 16 (ICGV 86855) |
| 29 | B 37c | SB | JL 24 x ICGV 87165 |
| 30 | B 31J | SB | JL 24 x ICGV 87165 |
| 31 | ICGV 86699 | VB | [(*A. batizocoi* x *A. duranensis*) x *A.hypogaea* (cv. NC 2)] CS 29 |
| 32 | ICGV 87165 | VB | [*A. hypogaea* var. *fastigiata* (PI 261942) x A. cardenasii] CS 9 |
| | **Others** | | |
| 33 | ICGV 86155 | VL | ICGS 30 x (TMV 10 x Chico F6) |
| 34 | TAG 24 | SB | TGS2 (TG 18A x M 13) x TGE1 (Tall x TG 9) |
| 35 | TG 26 | SB | BARCG 1 (gamma-rays mutant of JL 24) x TG 23 (TGS 2 x TGE 1) |
| 36 | Mutant 110 | VL | EMS mutant of VL 1 |

[a] Botanical variety: VL-Valencia, SB-Spanish bunch, VB-Virginia bunch, VR-Virginia runner; [b] DER is an intermediate type

was calculated as, PIC = 1 - $p^2$ - $q^2$ where, p = band frequency, q = no-band frequency [12], while for each primer, it was calculated by averaging for all the bands amplified by a primer.

## Results and discussion

### *Morphological diversity*

Principal component analysis based on 17 morphological characters grouped 36 genotypes into six clusters as determined by the 11 principal components, which contributed up to 95 percent of the variation (Table 2). The Virginia types comprising natural types (Chitra, M 13, S 230 and Mardur Local), mutants (NLM, VB 2, VB 8, VB 9, VR 1, VR 5, VR 8, JLVR 1 and JLVR 2) and interspecific derivatives (ICGV 86699 and ICGV 87165) were grouped into clusters I, III and V. DER was also grouped into cluster V. All the Valencia types consisting of natural types (PI 393516 and NCAc 17090), mutants (VL 1, VL 2 and VL 3) and ICGV 86155 were grouped into cluster IV, except Kopergaon3, which was grouped into cluster VI along with Spanish types. The Spanish types comprising natural types (TMV 2, JL 24 and KRG 1), mutants (SB 2, SB 3 and SB 6), TAG 24 and TG 26 were grouped into cluster VI. The Spanish and Valencia types showing resistance to diseases (late leaf spot and rust) were grouped into cluster II. Thus, genotypes belonging to a specific botanical type grouped together irrespective of their source of origin (natural types, mutants of DER or others, intra and interspecific derivatives) with few exceptions. For example, Virginia type mutants of JL 24 (JLVR 1 and JLVR 2) and DER (VB 8 and VB 9), Virginia type interspecific derivatives (ICGV 86699 and ICGV 87165) were grouped together with natural Virginia types (Chitra and M 13). Similarly, Virginia type Narrow leaf mutant (NLM) of TMV2 was grouped with Virginia type mutants of DER (VB2 and VB8). Similarity between the natural and mutant categories based on the morphology was evident. None of the clusters combined genotypes belonging to two different subspecies (ssp. *hypogaea* and ssp. *fastigiata*). Inter-cluster distances also confirmed the grouping of genotypes belonging to different botanical types into different clusters. The Valencia types (cluster IV) and Spanish types (cluster VI) were farthest from Virginia types (cluster I, V and III) but Valencia types (Cluster IV) were closer to Spanish types (cluster VI) (Table 2). The clusters belonging to the same botanical type showed least inter-cluster distances.

### *Native proteins*

The native protein profile of 36 genotypes revealed a total of 23 bands ($R_m$ = 0.029 to 0.971) out of which, only seven bands ($R_m$ = 0.259, 0.484, 0.507, 0.554, 0.557, 0.607 and 0.646) were polymorphic (30.43 per cent) displaying eight banding patterns (Table 3). None of the patterns could be specifically associated with any subspecies or botanical group. However, DER and some of its mutants (VL 2, VL 3, SB 2, SB 3, SB 6, VB 9 and VR 8), JL 24 and its mutants (JLVR 1, JLVR 2) and TMV2 and its mutant NLM could be distinguished using native protein profile. The dendrogram revealed eight clusters with similarity coefficient ($S_{ij}$) ranging from 0.86 to 0.97 (Fig. 1). PI 393516, JL 24, GPBD 4 and ICGV 87165 distinctly formed individual clusters due to their unique pattern. Most of the genotypes were grouped into four major groups wherein some botanical type was predominating with intermixing of other botanical types.

### *GOT isozyme*

The GOT isozyme analysis in hypocotyl tissue of the 36 genotypes revealed a total of five bands ($R_m$ = 0.479, 0.536, 0.598, 0.679 and 0.895; $1^{st}$ to $5^{th}$ band, respectively) (Fig. 2a). Depending on the presence or

**Table 2.** Distribution of 36 genotypes based on the principal component analysis of 17 morphological traits

| Cluster No. | Genotypes | Inter cluster distance | | | | |
|---|---|---|---|---|---|---|
| | | II | III | IV | V | VI |
| I | NLM, VB 2, VB 8 | 4.911 | 3.663 | 5.453 | 4.195 | 4.605 |
| II | Mutant 28-2, GPBD 4, B 37c, B 31J, Mutant 110 | - | 4.275 | 4.088 | 4.830 | 3.307 |
| III | Chitra, M 13, VB 9, VR 8, JLVR 1, JLVR 2, ICGV 86699, ICGV 87165 | - | - | 4.119 | 3.507 | 4.580 |
| IV | PI 393516, NCAc 17090, VL 1, VL 2, VL 3, ICGV 86155 | - | - | - | 5.240 | 3.558 |
| V | S 230, Mardur Local, DER, VR 1, VR 5 | - | - | - | - | 4.862 |
| VI | Kopergaon 3, TMV 2, JL 24, KRG 1, SB 2, SB 3, SB 6, TAG 24, TG 26 | - | - | - | - | - |

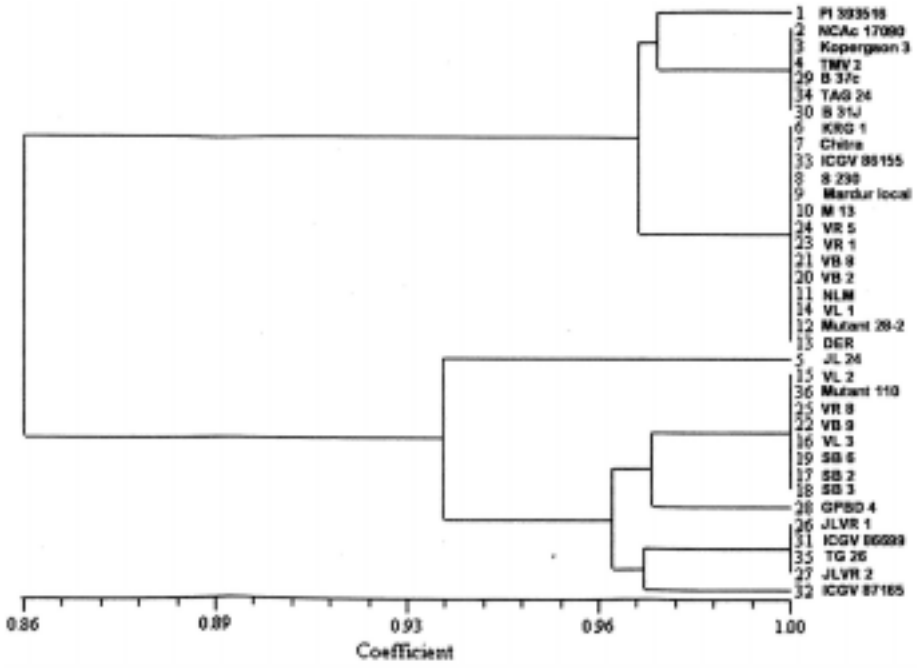


**Fig. 1. Dendrogram based on the Hypocotyl native protein profile; coefficient is Similarity coefficient ($S_{ij}$)**



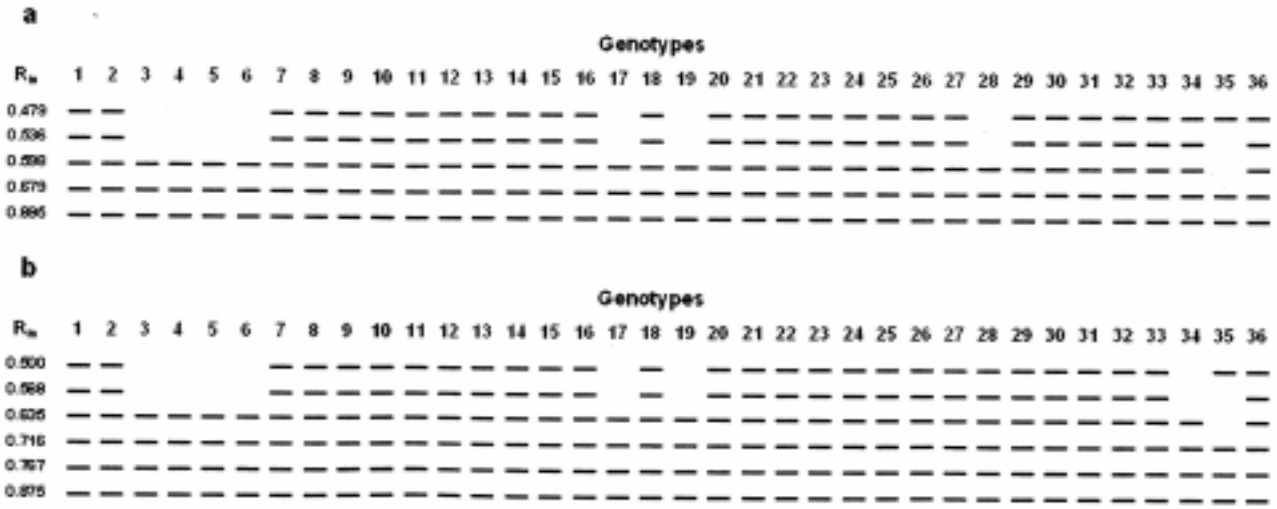


**Fig. 2. Glutamate oxaloacetate transaminase (GOT) isozyme profile in a) hypocotyls b) flowers; Relative mobility ($R_m$) value of the bands; Lanes/ Genotypes 1-36 as in Table 1**

absence of first, second and third bands, three banding patterns were observed. Pattern I had all the five bands, pattern II showed the absence of first and second bands, while pattern III showed the absence of second and third bands, respectively. All the Virginia types comprising natural types (Chitra, S 230, Mardur Local and M 13), mutants (NLM, VB 2, VB 8, VB 9, VR 1, VR 5, VR 8, JLVR 1 and JLVR 2) and interspecific derivatives (ICGV 86699 and ICGV 87165) showed pattern I. Similarly, all the Valencia types consisting of natural types (PI 393516 and NCAc 17090), mutants (Mutant 28-2, VL 1, VL 2, VL 3 and Mutant 110) and ICGV 86155 showed pattern I, except Kopergaon3, which displayed pattern II. All the Spanish types comprising natural types (TMV 2, JL 24 and KRG 1), mutants (SB 2 and SB 6) and interspecific derivative (GPBD 4) showed pattern II, except SB 3, B37c, B31J and TAG 24 which showed pattern I. TG 26 showed unique pattern III, which was not found in any other genotype under study. Natural types and mutants belonging to their respective botanical variety showed similar GOT isozyme pattern indicating that they are highly specific to the botanical types.

**Table 3.** Distribution of genotypes based on the hypocotyl native protein profile

| Electrophoretic pattern | Genotypes |
|---|---|
| I | PI 393516 |
| II | NCAc 17090, Kopergaon 3, TMV 2, B 37c, B 31J, TAG 24 |
| III | KRG 1, Chitra, S 230, Mardur Local, M 13, NLM, DER, Mutant 28-2, VL 1, VB 2, VB 8, VR 1, VR 5, ICGV 86155 |
| IV | JL 24 |
| V | VL 2, VL 3, SB 2, SB 3, SB 6, VB 9, VR 8, Mutant 110 |
| VI | GPBD 4 |
| VII | JLVR 1, JLVR 2, ICGV 86699, TG 26 |
| VIII | ICGV 87165 |

The GOT isozyme analysis in flowers of the 36 genotypes showed an additional monomorphic band compared to hypocotyls revealing a total of six bands ($R_m$ = 0.500, 0.568, 0.635, 0.716, 0.767 and 0.875; 1$^{st}$ to 6$^{th}$ band, respectively) (Fig. 2b). The additional band found in flowers was the fifth band ($R_m$= 0.767), which is between fourth and fifth band found in hypocotyl tissue. Depending on the presence or absence of first, second and third bands, three banding patterns were observed. Pattern I had all the six bands, pattern II showed the absence of first and second bands, while pattern III showed the absence of second and third bands, respectively. In total, pattern I had six bands, while pattern II and III had four bands each. GOT isozyme pattern in flowers was also highly specific to botanical types as observed in the hypocotyl tissue. All the Virginia types comprising natural types (Chitra, S 230, Mardur Local and M 13), mutants (NLM, VB 2, VB 8, VB 9, VR 1, VR 5, VR 8, JLVR 1 and JLVR 2) and interspecific derivatives (ICGV 86699 and ICGV 87165) showed pattern I. Similarly, all the Valencia types consisting of natural types (PI 393516 and NCAc 17090), mutants (Mutant 28-2, VL 1, VL 2, VL 3 and Mutant 110) and ICGV 86155 showed pattern I, except Kopergaon 3, which displayed pattern II. All the Spanish types comprising natural types (TMV 2, JL 24 and KRG 1), mutants (SB 2 and SB 6) and TAG 24 showed pattern II, except SB 3, B37c, B31J and GPBD 4 which showed pattern I. Like in hypocotyls, TG 26 showed unique pattern with absence of second and third bands in flowers and was not observed in any other genotype under study.

TAG 24 showed the banding pattern specific to Virginia/ Valencia types in hypocotyls and that of Spanish type in flowers. But, GPBD 4 showed the banding pattern specific to Spanish type in hypocotyls and that of Virginia/ Valencia types in flowers. The Virginia type mutants of JL 24 (JLVR 1 and JLVR 2), TMV 2 (NLM) and DER (VB 2, VB 8, VB 9, VR 1, VR 5 and VR 8) showed pattern similar to natural Virginia types (Chitra, Mardur Local, S 230, M 13) in both hypocotyls and flowers suggesting the similarity in the origin of isozyme diversity in natural and mutant categories.

*RAPD assay*

Thirty genotypes comprising natural and mutant categories belonging to different botanical groups *viz.*, Valencia (5), Spanish (12) and Virginia bunch/ runner (13) were subjected to RAPD assay using 21 primers. Out of 271 fragments amplified by 21 primers in 30 genotypes, 104 were polymorphic (38.38%) (Table 4).

**Table 4.** Polymorphism revealed by RAPD assay of 21 primers

| S.No. | Primer | No. of amplified fragments | No. of polymorphic fragments | Percent Polymorphism | PIC[a] |
|---|---|---|---|---|---|
| 1 | OPK 10 | 13 | 1 | 7.69 | 0.28 |
| 2 | OPK 12 | 10 | 0 | 0.00 | 0.00 |
| 3 | OPK 13 | 11 | 1 | 9.09 | 0.13 |
| 4 | OPK 14 | 12 | 2 | 16.67 | 0.27 |
| 5 | OPK 15 | 13 | 1 | 7.69 | 0.18 |
| 6 | OPK 16 | 12 | 1 | 8.33 | 0.06 |
| 7 | OPK 17 | 14 | 4 | 28.57 | 0.08 |
| 8 | OPK 18 | 16 | 9 | 56.25 | 0.29 |
| 9 | OPK 19 | 9 | 1 | 11.11 | 0.42 |
| 10 | OPF 20 | 16 | 5 | 31.25 | 0.26 |
| 11 | OPAT 1 | 12 | 9 | 75.00 | 0.20 |
| 12 | OPAT 2 | 16 | 11 | 68.75 | 0.21 |
| 13 | OPAT 3 | 11 | 5 | 45.45 | 0.21 |
| 14 | OPAT 4 | 15 | 8 | 53.33 | 0.28 |
| 15 | OPAT 5 | 15 | 2 | 13.33 | 0.13 |
| 16 | OPAT 6 | 16 | 10 | 62.50 | 0.30 |
| 17 | OPAT 7 | 16 | 6 | 37.50 | 0.25 |
| 18 | OPAT 8 | 18 | 11 | 61.11 | 0.22 |
| 19 | OPAT 9 | 11 | 6 | 54.55 | 0.40 |
| 20 | OPA 15 | 8 | 6 | 75.00 | 0.25 |
| 21 | OPA 18 | 7 | 5 | 71.43 | 0.36 |
| | Total | 271 | 104 | - | - |
| | Average | 12.90 | 4.95 | 38.38 | 0.23 |

[a]PIC – Polymorphism Information Content

On an average, 13 bands per primer were amplified and 4.95 bands per primer were polymorphic. Four to seven major fragments were amplified in any given sample along with a number of bands of lesser intensity. The polymorphism per primer ranged from 7.69 (OPK10 & OPK15) to 75 percent (OPAT1 and OPA15). Some of the primers namely, OPAT1, OPA15, OPA18, OPAT2, OPAT6, OPAT8, OPK18, OPAT9, OPAT4 and OPAT3 showed moderate level of polymorphism. The PIC values for primers ranged from 0.06 (OPK16) to 0.42 (OPK19) with an average of 0.23 (Table 4).

The primer OPAT2 amplified a specific amplicon (PIC=0.49) which was characteristically present in Virginia types (Chitra, S 230, M 13, NLM, VB 9, VR 8, JLVR 1, JLVR 2) with exceptions like VB 2, VB 8, VR 1 and VR 5 in which it was absent (Fig. 3a). It was also present in Valencia types (PI 393516, NCAc 17090, Mutant 28-2, VL 1, VL 2, VL 3 and Mutant 110). The characteristic amplicon was specifically absent in Spanish types (TMV 2, KRG 1, SB 2, SB 6, GPBD 4, ICGV 87165, TAG 24 and TG 26) with exceptions like JL 24 and SB 3 in which it was present. The amplicon was also absent in DER which is an intermediate type. The primer OPAT6 amplified a specific amplicon (PIC=0.44), which was characteristically present in Spanish types (TMV 2, JL 24, KRG 1, SB 2, SB 3, SB 6, GPBD 4, ICGV 87165, TAG 24 and TG 26) (Fig. 3b). This was also present in Valencia types (PI 393516, NCAc 17090, Mutant 28-2, VL 1, VL 3 and Mutant 110) with VL 2 as an exception. The characteristic amplicon was absent in Virginia types (Chitra, S 230, M 13, NLM, VB 2, VR 1, VR 8, JLVR 1 and JLVR 2) with exceptions like VB 2, VB 9 and VR 5 in which it was present. The amplicon was also present in DER. At least for natural types, it is possible to clearly distinguish both Virginia and Valencia types from Spanish types by the presence of specific fragment amplified by OPAT2; Spanish and Valencia types can be distinguished from Virginia types by the presence of specific fragment amplified by OPAT6.

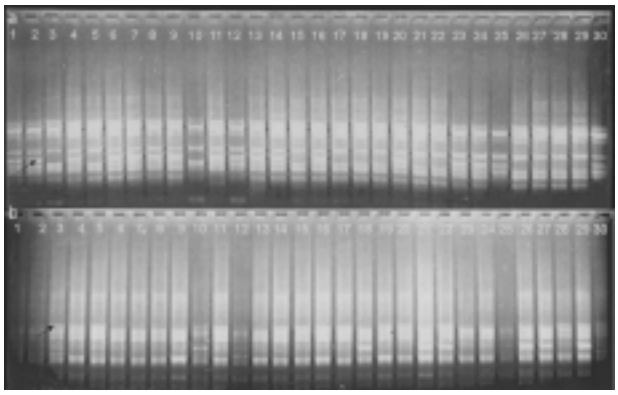

**Fig. 3. RAPD profile of the primer a) OPAT2 b) OPAT6; arrow mark indicates specific amplified fragment; Lanes/Genotypes: 1.PI 393516, 2.NCAc17090, 3.TMV 2, 4.JL 24, 5.KRG 1, 6.Chitra, 7.S 230, 8.M 13, 9.NLM, 10.DER, 11.Mutant 28-2, 12.VL 1, 13.VL 2, 14.VL 3, 15.SB 2, 16.SB 3, 17.SB 6, 18.VB 2, 19.VB 8, 20.VB 9, 21.VR 1, 22.VR 5, 23.VR 8, 24.JLVR 1, 25.JLVR 2, 26.GPBD 4, 27.ICGV 87165, 28.TAG 24, 29.TG 26, 30.Mutant 110**

The dendrogram revealed three distinct clusters at similarity coefficient ($S_{ij}$) of 0.87, 0.92 and 0.94, respectively (Fig. 4). Natural Valencia types (PI 393516, NCAc 17090) were distinctly clustered from all others. Natural Virginia types (Chitra, S 230, M 13) were distinctly clustered together. Most of the Spanish types (TMV 2, JL 24, KRG 1, GPBD 4, TG 26, TAG 24) were solitarily clustered. Some of the genotypes and their mutants belonging to different botanical groups were quite distantly placed like TMV 2 and NLM, JL 24 and JLVR 1, JLVR 2. Mutants derived from the same source belonging to the same botanical type (Mutant 28-2 and Mutant 110) were clustered together. DER and its mutant VL 1 were also clustered together. However, the genotypes belonging to natural and mutant categories could not be distinctly clustered according to the botanical groups to which they belong.

Mutants derived from DER along with other mutants and natural types exhibited similarity among the natural and mutant categories when assessed for morphological, biochemical (native proteins and GOT isozyme) and molecular (RAPD assay) diversity. This suggests that mutations have played a key role in creating enormous diversity in terms of different subspecies and botanical types of groundnut in Nature as well. The possible role of spontaneous mutations in the evolution of abundant morphological variation in groundnut is also evident from the various subspecific changes brought about by induced mutations in earlier breeding programmes [13-15]. Unusual features like high frequency of multiple mutations [16], homozygous mutations [17] and a large number of mutants from a single aberrant cytological variant [18] were also observed in some earlier mutation experiments [3]. The wide genetic diversity of groundnut existing in Africa has been indicated to have come from a single source, as the original material introduced into Africa mainly consisted of *fastigiata* type [19].

Treatment of DER with mutagens like EMS, gamma rays and 5-azacytidine might have caused a

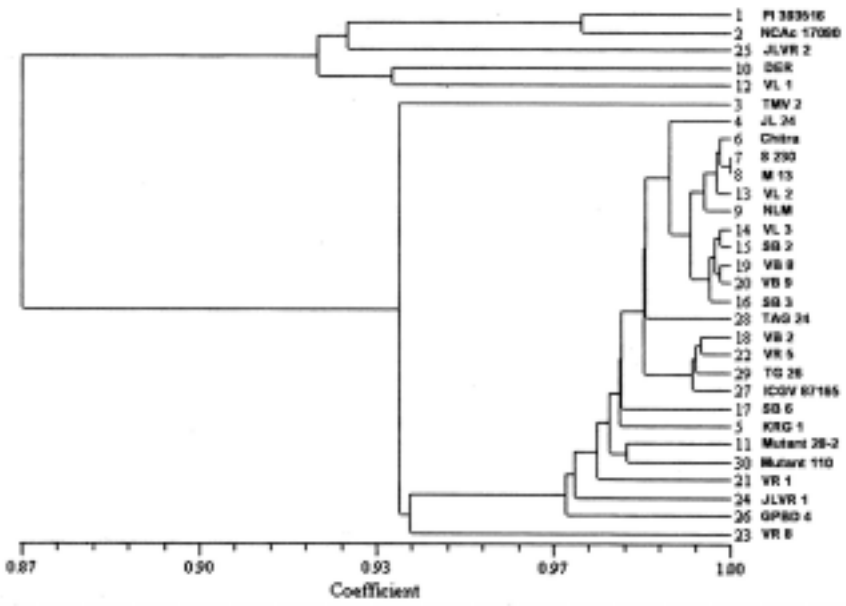


**Fig. 4. Dendrogram based on the RAPD profile of 21 primers; Coefficient is Similarity coefficient ($S_{ij}$)**



genome shock or stress [20] resulting in a genome-wide epigenetic modifications creating 'epialleles' (involving non-DNA sequence changes) giving rise to 'epimutants' resembling different botanical types. There is probably less chance for the groundnut genome to evolve with simple classical mutations to the currently observed levels of variability, as groundnut is an inbreeder and an allopolyploid with extensive gene buffering. Behaviour of the DER derived mutants and several unusual features are indicative of non-Mendelian turnover mechanisms. They were observed at a very high frequency in a single generation by far exceeding the rate of mutational events giving rise to new alleles and their reversion rate was by far higher compared to classical mutations [3]. They are probably 'epimutants' as they are more flexible and relatively less stable [21].

The possible involvement of epigenetic changes in bringing about enormous diversity is supported by the genetic behaviour of these mutants as well as their response to 5-azacytidine (a demethylating agent). 'TMV 2' (a Spanish bunch variety) on mutagenesis with EMS (0.2%) had earlier resulted in a 'Narrow leaf mutant' (NLM) with a shift to Virginia bunch type [13]. Genetic studies indicated that under the assumption of independence, TMV 2 and its mutant differ for at least 22 genes for the eight morphological characters studied for which they differ [22]. As they also differ for several other characters which were not assessed, they are expected to differ for many more genes. The multiple gene differences between TMV 2 and its mutant indicated that NLM is not just a simple mutation. TMV 2 and NLM were treated with 5-azacytidine (0.5 mM for 24 hours) to study the possible involvement of DNA methylation. NLM on treatment with 5-azacytidine showed a shift from Virginia bunch to Virginia runner with 'broad leaf' like TMV 2. Most of them bred true and some segregated for broad and narrow leaf types [23]. The results indicated that NLM is probably in 'hypermethylated' condition, which on treatment with 5-azacytidine might have resulted in 'broad leaf types' which are in 'hypomethylated' condition but, TMV 2 did not respond to 5-azacytidine because it may be already in 'hypomethylated' condition. This indicates the possible involvement of 'epigenetic determinants' in the transformation of botanical types. Polyploids are known to respond epigenetically to 5-azacytidine [24]. Epigenetic gene silencing coupled with allopolyploid formation has been reported to be genomically global and phylogenetically wide spread [24-26]. DNA methylation and histone modifications have been found to serve as epigenetic marks in the maintenance of silencing of genes in allopolyploids [24]. Differential epigenetic modifications of homeologous genes in polyploids have been reported to play an important role in leading to natural variation and evolutionary opportunities for adaptive selection and domestication [27].

GOT isozyme is the most prominent biochemical marker associated with subspeciation in groundnut [28]. Both direct and reciprocal crosses involving TMV 2 and NLM showed 'paternal inheritance' of GOT isozyme [23] as earlier reported [29]. In the $F_2$ generation, they segregated into two parental types (presence or absence of two slowest bands) in both direct and reciprocal crosses. This further suggests that either they are expressed (presence) or silenced (absent) depending on the epigenetic status of the gene/s controlling GOT isozyme. 'Genomic / parental imprinting' seems to be the most probable cause as it showed the expression (presence) or silencing (absence) of two slowest GOT bands without any new bands revealing absence of intra or inter-allelic interactions. This is also supported by the fact that the segregation of GOT did not fit any genotypic model. Imprinting has been reported

in plants for genes controlling endosperm development in maize [30] and MEDEA (*MEA*) gene encoding polygroup protein regulating cell proliferation in *Arabidopsis* [31]. Furthermore, a chromatin-remodeling factor has been implicated as a novel transacting regulator of imprinting [32]. The most striking feature of imprinted genes is that the active and inactive parental alleles of the same DNA sequence coexist within individual cells. Parental alleles of imprinted genes display differential DNA methylation capable of regulating and transmitting the repressed and active states of imprinted genes to the gametes. Homeologous alleles for some loci in cotton allotetraploids have shown alternate expression patterns in different tissues [33] like tissue specific expression of GOT isozyme in TAG 24 and GPBD 4 in the present study.

Despite the existence of substantial diversity among the cultivated groundnut genotypes for various morphological, physiological and agronomic traits, very limited DNA polymorphism has been shown in cultivated groundnut [34,35] as observed in the present study by RAPD assay. If the morphological diversity is predominantly based on the 'epigenetic determinants', the conventional DNA markers based on sequence differences are expected to have limited potential to be polymorphic. Alternatively, markers that could differentiate the 'epigenetic states' would be more appropriate to uncover the basis of morphological diversity. To ascertain the 'epigenetic basis' of creation of diversity in groundnut, DER derived mutants need to be subjected to techniques like MSAP (Methylation sensitive amplification polymorphism), CRED-RA (Coupled restriction enzyme digestion and random amplification) etc. to detect the genome-wide cDNA-AFLP and cDNA-SSCP (Single strand confirmation polymorphism) to unravel the genes associated with genetic differentiation by studying their expression status i.e. expressed or silenced. Such studies would eventually shed more light on the 'epigenetic' nature of origin of diversity in groundnut and will have implications to its genetic improvement.



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