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Molecular diversity and association of simple sequence repeat markers with kernel mass in cultivated groundnut (*Arachis hypogaea* L.)

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Abstract

Groundnut yield can be further enhanced by improving pod and kernel size vis-a-vis mass. Marker assisted breeding will be an ideal option for directed improvement of hundred kernel mass. A study was undertaken to detect molecular diversity using 35 SSRs in 12 mutant genotypes, developed through chemical mutagenesis, from an interspecific large kernel size pre-breeding line and to identify markers associated with kernel mass. SSRs yielded an average of 3.57 polymorphic bands per primer. Average polymorphism and PIC were 64.95% and 0.62, respectively. Cluster analysis revealed two main clusters separated at 61% Jaccard's similarity coefficient. Vast of the genotypes were grouped into single cluster, confirming common pedigree of these genotypes. AMOVA among 12 mutant genotypes and their parent detected 15% of total variation associated with kernel mass. K-W ANOVA detected significant association of five SSRs with kernel mass. Among these associated primers, TC3A12 and TC9H09 accounted for 28% and 12% of phenotypic variation due to kernel mass and were associated with major QTLs. Out of these two associated primer, TC3A12 differentiated genotypes with higher kernel mass from genotypes with lower kernel mass by amplifying a band of approximately of 450bp. Thus association of TC3A12 primer with a major QTL of kernel mass was further validated in genotypes with diverse background. The TC3A12 primer discriminated genotypes with higher kernel mass from genotype with lower kernel mass by amplifying the band of 400bp among genotypes with higher kernel mass.

Keywords: Groundnut; bulked segregant analysis; molecular marker; hundred kernel mass; simple sequence repeat.
Abbreviation: AFLP: Amplified fragment length polymorphism, AMOVA:Analysis of molecular variance, ANOVA: Analysis of variance, BSA: Bulked segregant analysis, CTAB: Cetyl Trimethyle ammonium bromide, DES: Diethyl sulfate, EMS: Ethyl methane sulfonate, HKM: Hundred kernel mass, HPS: Handpicked selection, ISSR: Inter-simple sequence repeat, MAS:Marker assisted selection, MI: Marker index, PCR: Polymerase chain reaction, PIC: Polymorphic information content, QTL: Quantitative trait loci, RAPD: Random amplified polymorphic DNA, SSR: Simple sequence repeat, UPGMA: Unweighted pair group method with arithmetic mean.

Introduction

Groundnut (*Arachis hypogaea* L.) is an important oilseed crop grown in approximately 24 m ha throughout the world (FAO statistical database, 2010). In groundnut, and other plant species, the majority of economically important agronomic characteristics are controlled in a quantitative fashion. Until recently, plant breeders have relied on phenotypic selection methods to improve specific quantitative traits. Due to effects of the environment on these traits, such methods can be expensive, time consuming and labour intensive. Breeding efforts to improve these traits could be more efficient and successful with the use of molecular marker and well saturated genomic map (Samizadeh et al., 2003; Varshney et al., 2005a, 2005b). The groundnut, which is used for direct consumption in domestic and international market, is generally referred as large seeded groundnut or hand picked selection (HPS) groundnut (Dwivedi and Nigam, 2005). A maximum count of 44 kernels per 25g of the materials (Reddi, 1988) or in other words 100 kernel mass of 57 gram or more is essential for a groundnut sample to qualify for grading as HPS groundnut. HPS groundnut fetches premium price due to its growing demand in international market. Hundred kernel mass is an important yield attributing trait as well as a physical attributes of HPS groundnut, often targeted to improve pod yield in groundnut. HKM in groundnut is polygenically controlled and can range from 15 to 110 grams per 100 kernels, directly associated with pod yield (Gomes and Lopez, 2005). Plant breeders have been successful in manipulating this trait, but the underlying genetic basis for HKM has not been elucidated. Information on the association between genetic markers and HKM should help breeders construct beneficial allelic combinations and accelerate the development of speciality cultivars and enhance pod yield in groundnut. Cultivated groundnut has been characterized with narrow genetic base and exhibits a low level of variation at the DNA level as revealed by using RAPD (Halward et al., 1991; Subramanian et al., 2000), ISSR (Raina et al., 2001), AFLP (Herselman 2003; Gimenes et al., 2002), and SSR markers (Halward et al., 1991; Paik-Ro et al., 1992; Kochert et al., 1996; He et al., 2005). Of the major DNA marker types, SSR marker has been the most successful at identifying molecular variation within the cultivated groundnut species (Hopkins and Casa, 1999; Ferguson et al., 2004; Mace et al., 2006) and good progress has been made in tagging economically-important traits in groundnut using RAPD, ISSR, SSR and SCAR markers (Garcia et al., 1995; Burow et al., 1996; Choi et al., 1999; Stalker and Mozingo, 2001; Herselman et al., 2004; Mondal et al., 2005; Yong et al., 2005; He et al., 2005; Mace et al., 2006; Burow et al., 2008; Selvaraj et al., 2009; Khedikar et al 2010; Gautami et al., 2011; Shirasawa et al.,

Table 1. Hndred kernel mass (g) of parent NRCGCS-281 during rabi-summer season (February to May) over locations and years.

Year	Junagadh Gujarat state	Dharwad Karantaka state	Vridhachalam Tamil Nadu state	Rahuri Maharashtra state	Shirgaon Maharashtra state	Mean
2008-09	71.0	73.0	89.0	83.0	65.0	76.0
2009-10	73.0	73.0	82.0	91.0	76.0	80.5
Mean	72.0	73.0	85.5	87.0	70.5	78.3

2012). In addition to, few genetic linkage maps have been developed using wild species (Halward et al., 1993; Burow et al., 2001; Garcia et al., 2005; Moretzsohn et al., 2005) as well as cultivated groundnut (Varshney et al., 2009; Ravi et al., 2010; Sujay et al., 2012). Selvaraj et al. (2009) first reported association of five SSRs with pod and kernel characters in groundnut, of which PM375 was associated with a major QTL for 100 seed mass. Association of seed weight with molecular markers was also reported in soybean, cowpea and mung bean and found that seed weight in these species is controlled by an orthologous genomic region in quantitative fashion (Maughan et al., 1996; Mian et al., 1996). The purpose of this research was to re-confirm QTL reported earlier and identify additional QTL if any for hundred kernel mass in groundnut.

Results

Phenotyping

In this study mutants with small and large kernel size were developed by chemical mutagenesis of NRCGCS-281, an advanced pre-breeding line with large kernel size and deep pod constriction (Fig. 1 and Table 1). Selected mutants of NRCGCS-281 were forwarded to M_6 generation by selfing for attaining homozygosis for pod and kernel traits. Phenotyping for HKM was done in $M_{5:6}$ progeny. Four large and eight small kernel size genotypes bred true along with NRCGCS-281 (Table 2) were subjected to molecular analysis using 35 SSRs. HKM of eight selected mutants with small kernel size were 25.7 g, 31.7 g, 33.8 g, 32.1 g, 37.8 g, 31.0 g, 38.7 g and 36.3 g, respectively with an average of 33.4 g. In contrast, HKM of four selected mutants with large kernel size were 68.1 g, 66.5 g, 63.4 g and 64.0 g, respectively with an average of 68.2 g across growing seasons. Thus average HKM of selected large kernel mutants was found approximately twice (68.2 g) the average HKM of selected small kernel mutants (33.4 g) (Table 2). Similarly, the kernel length of genotypes with higher HKM was observed approximately twice of the kernel length of genotypes with lower HKM (data not shown) (Fig. 1a, 1b, 2a and 2b).

Genotyping

Primer pairs used in the study yielded a total of 170 fragments, of which 125 were polymorphic, with an average of 3.57 polymorphic fragments per primer. Twenty eight SSRs were polymorphic, while seven were monomorphic (Table 4). Polymorphism ranged from zero to 100% with an average of 62.0%. Higher polymorphism (>50%) was observed in case of 23 primers. Number of amplified fragments ranged from 1 to 15 per locus. Above average number of alleles per locus was observed in 11 primers. Among polymorphic primers, PM375 produced the highest (15) number of alleles.

Genetic diversity

The PIC value of SSRs ranged from zero to 0.91 with an average of 0.63. The MI value of primers ranged from zero to

Fig 1. Pod size and shape of NRCGCS-281(a) and a small kernel mutant (b)

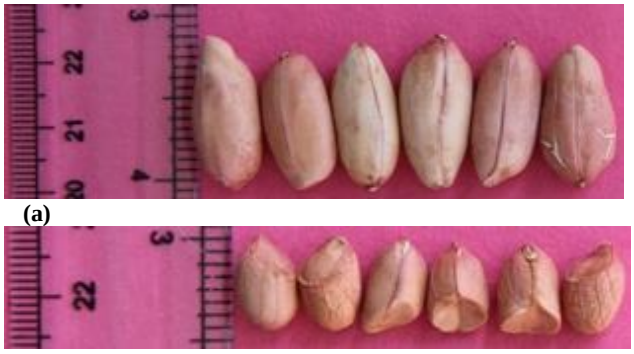
85.5 with an average value of 43.4. Out of these polymorphic primers, TC11B11, TC2B01, TC4G02, TC9B08, TC3A12, TC11B04, PM375, PM478, TC9H09, and TC3D04 had higher number of alleles per locus along with higher polymorphic per cent, PIC content and MI value. These selected primers can be considered as highly informative in revealing the genetic diversity and partitioning genetic variation in cultivated groundnut. The dendrogram was constructed through SHAN clustering and UPGMA analysis. Twenty-eight polymorphic primers discriminated 13 genotypes into two clusters. All genotypes were grouped into single cluster (denoted as cluster-I) except NRCGCS-281-7 (Fig. 3). Cluster-I was further subdivided into two sub clusters (Ia and Ib). Sub-cluster-Ia contained two genotypes while sub-cluster-Ib contained 10 genotypes. Cluster-II contained single genotype. Maximum similarity (84%) was observed between genotypes NRCGCS-281-17-3 and NRCGCS-281-25-2, while minimum similarity (61%) was observed between genotypes NRCGCS-281-7 and NRCGCS-281-25-2, or NRCGCS-281-17-3. Thus, genotypes NRCGCS-281-17-3 and NRCGCS-281-25-2 were closely related to each other, while parental genotype NRCGCS-281 was distantly related to NRCGCS-281-17-3, and NRCGCS-281-25-2.

Marker trait association

AMOVA among two groups detected 15% ($P<0.025$) of total variation associated with higher HKM (Table 5). Kruskal-Wallis ANOVA detected the significant association of six primers (TC3A12, TC4G02, TC9B08, PM478, TC9H09, and TC3D04) with higher HKM (Table 6). These six primers had higher number of alleles per locus along with higher polymorphic per cent, PIC content and MI value and showed significant association with HKM by simple regression analysis. Among these six primers, TC3A12 and TC9H09 explained 28.2% ($P=0.006$) and 12.1% ($P=0.003$) of

Table 2. Hundred kernel mass of NRCGCS-281 and its 12 mutants.

Sr. No.	Mutants	HKM (g)	Kernel size denoted
1	NRCGCS-281	79.0	Large
2	NRCGCS 281-2	68.1	Large
3	NRCGCS 281-21-2	66.6	Large
4	NRCGCS 281-25-11	63.4	Large
5	NRCGCS 281-40-3	64.0	Large
	Mean	68.2	
6	NRCGCS 281-5-1	25.7	Small
7	NRCGCS 281-6	31.7	Small
8	NRCGCS 281-7	33.9	Small
9	NRCGCS 281-8	32.1	Small
10	NRCGCS 281-14-1	37.9	Small
11	NRCGCS 281-17-1	31.0	Small
12	NRCGCS 281-17-3	38.7	Small
13	NRCGCS 281-25-2	36.3	Small
	Mean	33.4	



(a)

(b)

Fig 2. Kernel size of NRCGCS-281 (a) and small kernel mutant (b)

phenotypic variations, respectively due to higher HKM. Out of these two, TC3A12 amplified an allele of approximately 400bp in genotypes with higher HKM which did not amplify in genotypes with lower HKM. Thus, TC3A12 was able to discriminate the pooled DNA of genotypes with higher HKM from the pooled DNA of genotypes with lower HKM.

Validation of marker trait association

The association of locus $TC3A12_{450}$ with HKM was further validated in genotypes having different background with an objective to test the reliability of the marker. For this purpose, four varieties with higher HKM and one variety with lower HKM of diverse background were used to check the presence of allele (Table 7). The TC3A12 primer amplified the 400bp allele in three varieties with higher HKM and did not amplify in variety with lower HKM (Fig. 5).

Discussion

Use of mutation induced variability in molecular marker analysis

Mutation breeding was used for improvement of plant characters and increasing genetic variability in various crops including groundnut. Deep pod constriction increases pod-loss at harvest and lowers market value of groundnut produce. Present study helped to improve pod and kernel traits of NRCGCS-281 through mutation breeding and to develop genotypes with higher HKM and without pod constriction (Fig. 6). Genetic variation, induced for pod and kernel size and shape through mutation, were used to identify molecular diversity and association of SSR markers with kernel mass. Characterization of mutants and molecular markers associated with resistance to pod rot diseases and aflatoxin contamination using RAPD and ISSR markers have been reported earlier in groundnut (Azzam et al., 2007).

SSRs and genetic diversity

DNA markers have been used to evaluate genetic diversity in different crops (Cooke, 1995; Azzam et al., 2007). Recently co-dominant markers, such as SSR and EST-SSR available in groundnut has greatly aided in diversity and genome studies in this crop. Vast of the primers, used in the study, were highly polymorphic producing higher number of alleles per locus. Primers amplified more than one locus in groundnut genotypes indicating loci duplication. This may be attributed to the presence of A-genome as well as B-genome in the allotertraploid cultivated groundnut. Amplification of more than one fragment by one pair of primer in tetraploid groundnut accessions has also been reported in earlier studies (Gimenes et al., 2007; Varshney et al., 2009; Hopkins and Casa, 1999). The PIC values derived from allelic diversity and frequency among the genotypes were not uniform among the SSR loci tested. The higher PIC value of primers could reveal maximum genetic information among genotypes under investigation. Majority of the primers, used in the study, had higher PIC value (>0.5). Such higher PIC value could be due to marker pre-selection with higher GC/CT repeats. Quantitative estimation of marker utility and detection of polymorphism have been depicted in terms of mean heterozygosity and MI (Powel et al., 1996). Hence, diversity revealed, based on PIC values, needs to be varified by additional measures, like polymorphic per cent, MI value and number of alleles amplified per locus prior assessing their informativeness. Thus, 10 out of 35 primers used in the study, were highly informative in revealing the genetic diversity and partitioning of genetic variation due to their higher number of alleles per locus as well as higher PIC and MI values. The dendrogram grouped vast of the genotypes into single cluster indicating lower level molecular diversity among genotypes, as genotypes were bred through mutation of single parent. Nevertheless, low level of polymorphism has been reported in cultivated groundnut by previous workers (Kochert et al., 1991; He and Prakash 1997; Moretzsohn et al., 2004; Mace et al., 2006).

Association SSRs with hundred kernel mass

The diversity revealed in this study was further used to identify SSR associated with higher kernel mass and to use in MAS. MAS has been proved to be a more efficient, accurate, and simpler strategy for selection of desired genotype (Kwon and kim, 2001). In this study, two SSRs (TC3A12 and TC9H09) were found associated with major QTLs for HKM in groundnut. SSRs linked with a trait and explaining more than 10% of total phenotypic variation (r^2) are considered to be major QTLs (Collard et al., 2005). QTLs identified in this study were different from those identified by Selvaraj et al., (2009). These additional QTLs would be of help in saturated linkage mapping and pyramiding alleles towards improving kernel size more precisely through MAS. BSA has been used as an alternative method to the traditional QTL analysis using

Table 3. SSR primers used in the study.

Sr. No.	Primers	Sequence	bp	Tm
1	AC1G11	F- CCTTTTTCTTTCAAGGCTCCTA	44	52.8^{O}C
		R- TTCGTAAATCAGAGGTGGTGAG		54.3 ^{O}C
2	AC1E11	F- CTTTGTCCATCTCTGCAACTCC	44	55.8 ^{O}C
		R- GCGCTCGAAGCTCTATGAATAA		54.9 ^{O}C
3	AC2A04	F- GATCACTCCAGATAATCAC	37	47.0 ^{O}C
		R- AAGGTTATCACTCACGTC		48.7 ^{O}C
4	AC2A09	F- ACTTGCTGAGTCTTGTCCCAAT	44	56.5 ^{O}C
		R- TCTCCAATGTTCACAATCAAGC		53.5 ^{O}C
5	AC2C02	F- CTTCGACGGAAGATCGTATTT	42	52.3 ^{O}C
		R- GGGTGCTATAATGGCTGAACT		54.7 ^{O}C
6	AC2C05	F- CAAGGAAGCGTGAATTGTTAG	43	51.9 ^{O}C
		R- TGTGGACTATGCTTGTCATGTT		54.4 ^{O}C
7	AC2C08	F- CTTCAGTGTGGTGTCTCTCGAC	44	57.1 ^{O}C
		R- CCTCTAACTTTTCCGGGTTCTT		54.6 ^{O}C
8	AC2C12	F- TATCGAGCCGAATATGAAT	40	48.0 ^{O}C
		R- GCAGGATTTTGTAATTGAGAG		49.4 ^{O}C
9	AC2F08	F- GATCACTCAAACCCTAATCCAA	41	52.4^{O}C
		R- AGGCTTCCAAATGCTCACT		54.4 ^{O}C
10	AC2H11	F- TCCTTTACTTGTGCAGTTGTGC	41	55.9 ^{O}C
		R- AAAACGCCATGTGGTGGAT		55.3 ^{O}C
11	AC3C02	F- TCTAACGCACACAAATCGAA	40	52.2 ^{O}C
		R-CTTGTACCTGCGCCATTCT		55.3 ^{O}C
12	AC3D07	F- TAGCTTCGATAACCAGGGAGAC	44	55.8 ^{O}C
		R- CCCTAACACTCGTTCATTCCTC		55.8 ^{O}C
13	AC3F05	F- GAGATTCGTATATGCCCTTA	46	48.7 ^{O}C
		R-GATCATAGATACAAGATAATCAAAGT		48.8 ^{O}C
14	AC3F07	F- TATCCTGCTTCAGCTCCCATT	41	56.0 ^{O}C
		R- CGGCCCAGCATTCTAAAGTA		54.8 ^{O}C
15	PM588	F-CCATTTTGGACCCCTCAAAT	42	53.1 ^{O}C
		R-TGAGCAATAGTGACCTTGCATT		54.7 ^{O}C
16	TC4D09	F- TTGTGCTCTGCTCTTGGTTG	40	55.7 ^{O}C
		R- CTTGCTGGAGGAAACACACA		55.4 ^{O}C
17	TC11B11	F- AGAGTAGGCAACATCCTTGGAA	44	55.9 ^{O}C
		R- ACATTAACAGCCACACCCTCTT		56.6 ^{O}C
18	TC2B01	F- TTGCAGAAAAGGCAGAGACA	44	54.6 ^{O}C
		R- GAAAGAAGCTAAGAAGGACCCATA		54.2 ^{O}C
19	TC2CO7	F-CACCACACTCCCAAGGTTTT	40	55.5 ^{O}C
		R-TCAAGAACGGCTCCAGAGTT		56.2 ^{O}C
20	TC2E05	F- GAATTTATAAGGCGTGGCGA	40	53.0 ^{O}C
		R- CCATCCCTTCTTCCTTCACA		54.3 ^{O}C
21	TC2E11	F- AAACTTGGACGTTGGCTTTG	40	54.1^{O}C
		R- CAGGTTCGGGTTCACACTTT		55.7 ^{O}C
22	TC3A12	F- GCCCATATCAAGCTCCAAAA	40	53.1 ^{O}C
		R- TAGCCAGCGAAGGACTCAAT		56.0 ^{O}C
23	TC3B05	F- GGAGAAAACGCATTGGAACT	40	53.5 ^{O}C
		R- TTTGTCCCGTTGGGAATAGT		53.9 ^{O}C
24	TC4G02	F- GATCCAACTGTGAATTGGGC	40	54.1 ^{O}C
		R- CACACCAGCAACAAGGAATC		54.3 ^{O}C
25	TC9B08	F- GGTTGGGTTGAGAACAAGG	41	53.6 ^{O}C
		R- ACCCTCACCACTAACTCCATTA		55.1 ^{O}C
26	TC9C06	F- CAAATGGCAGAGTGCGTCTA	38	55.2 ^{O}C
		R- CCCTCCTGACTGGGTCCT		58.7 ^{O}C
27	TC9H08	F- GCCAAAGGGGACCATAAAC	40	53.8 ^{O}C
		R- TCCATCTTCCATCTCATCCAC		54.0 ^{O}C
28	TC11B04	F- GATCTGAAGGCTCTGATACCAT	44	53.9 ^{O}C
		R- GATCTCAACCAGAACAGTATGC		53.3 ^{O}C
29	TC11C06	F- TCCAACAAACCCTCTCTCTCT	42	55.2 ^{O}C
		R- GAACAAGGAAGCGAAAAGAA		51.1 ^{O}C
30	PM375	F- CGGCAACAGTTTTGATGGTT	39	54.2 ^{O}C
		R- GAAAAATATGCCGCCGTTG		52.7 ^{O}C
31	PM478	F-GTCGTGCAGGTCAAAGTG	38	57.0 ^{O}C
		R-TTAAGATGGGTGCCTGCAAT		54.6 ^{O}C
32	TC9H09	F- TTAGCGACAAAGGATGGTGAG	43	54.8 ^{O}C
		R- TAGGGACGAAAATAGGGACTGA		54.9 ^{O}C
33	TC3EO5	F-CACCACTTGAGTTGGTGAGG	40	55.7 °C
		R-CTTCTTCTTCTCCCGCAATG		53.5 °C
34	TC3DO4	F- TTTCGTCATTTCAGCTCCTC	41	55.5°C
		R-TTCAGCCTAGAGCGGTATTCA		52.6°C
35	TC2CO3	F- AGACGTGAGTGCTTGGTTCA	40	56.6°C
		R- CAGCCTAGAGCCGAATTCAC		55.6°C

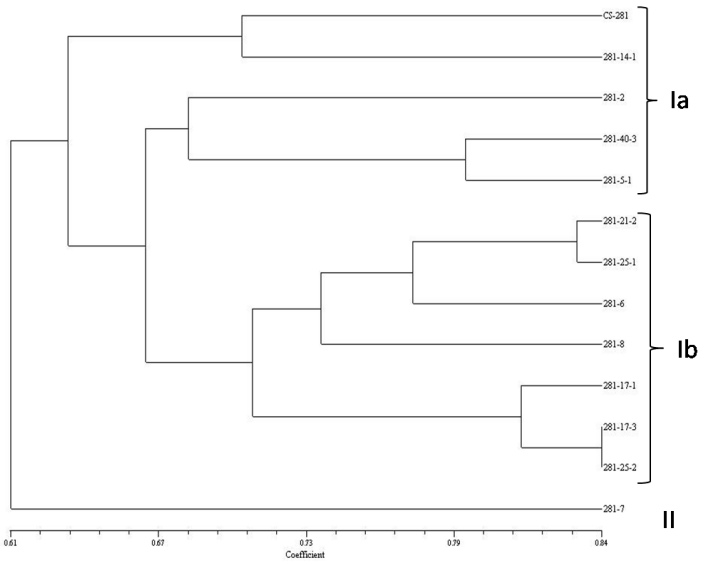


Fig 3. UPGMA tree showing relationship among 13 peanut genotypes based on 35 SSR loci. Cluster I includes vast of the genotypes and cluster II includes single genotype. Genotypes are presented with mutant number.

biparental segregation populations for identifying markers linked to traits of interest (Sun et al., 2003; Mondal and Badigannavar, 2010; Selvaraj et el., 2009). Though BSA is generally used to tag genes controlling simple traits, but the method may also be used to identify markers linked to major QTLs (Wang and Peterson, 1994). In the present study, BSA permitted identification of the QTLs for the higher HKM in groundnut both by permitting analysis in absence of a linkage map and by reducing the degree of effort needed to identify associations between markers and phenotypes.

Materials and Methods

Plant materials

The NRCGCS-281 is an interspecific pre-breeding groundnut genotype with deep pod constriction and large kernel size with HKM of about 79 g (Table 1, Fig.1a and 2a). Kernels of NRCGCS-281 was treated with the 1:1 (v/v) solution of 0.25% ethyl methane sulfonate and 0.25% dietyl sulfate for eight hours followed by through washing under tap water before sowing. Small and large kernel size genotypes were isolated in M_2 generation and forwarded to M_6 generation. Phenotyping for HKM was done in $F_{5:6}$ progeny. Selected four mutant genotypes with large HKM and eight mutant genotypes with small HKM, bred true, along with NRCGCS-281 (Table 2) were subjected to molecular analysis using 35 SSRs (Table 3).

Isolation of DNA

Genomic DNA was extracted from leaf samples of field grown plants following CTAB method (Doyle and Doyle, 1987) and the quantity was checked by 0.8% (W/V) Agarose gel electrophoresis.

PCR amplification and Gel Electrophoresis

The PCR mixtures (8µl) contained 0.5 µl (50ng) genomic DNA, 2.5U of Taq DNA polymerase, 0.75µl of 10X Taq Buffer (Genei, Banglore, India), 0.5µl dNTPS (10mM)

Table 4. Polymorphism detected by the use of 35 SSRs on 13 groundnut genotypes.

Sr.No.	Primers	Number of allele amplified	Polymorphic Per cent	PIC value	MI value
1	AC1G11	3	66.66%	0.61	40.66
2	AC1E11	3	100.00%	0.63	63.54
3	AC2A04	4	100.00%	0.74	74.00
4	AC2A09	3	100.00%	0.67	67.00
5	AC2C02	2	50.00%	0.49	24.57
6	AC2C05	3	100.00%	0.69	69.00
7	AC2C08	2	100.00%	0.32	32.00
8	AC2C12	3	33.33%	0.67	22.33
9	AC2F08	4	25.00%	0.68	17.00
10	AC2H11	4	75.00%	0.74	55.50
11	AC3C02	2	50.00%	0.40	20.00
12	AC3D07	4	100.00%	0.68	68.00
13	AC3F05	3	66.66%	0.59	39.32
14	AC3F07	1	0.00%	0.00	00.00
15	PM588	4	100.00%	0.68	68.00
16	TC4D09	1	100.00%	0.00	00.00
17	TC11B11	7	100.00%	0.78	78.00
18	TC2B01	6	83.33%	0.78	62.49
19	TC2C07	4	0.00%	0.75	00.00
20	TC2E05	6	66.66%	0.78	51.99
21	TC2E11	6	50.00%	0.77	38.50
22	TC3A12	14	71.00%	0.92	65.32
23	TC3B05	1	0.00%	0.50	00.00
24	TC4G02	10	90.00%	0.91	81.90
25	TC9B08	8	100.00%	0.85	85.45
26	TC9C06	2	0.00%	0.66	00.00
27	TC9H08	1	0.00%	0.00	00.00
28	TC11B04	7	85.71%	0.81	69.45
29	TC11C06	3	100.00%	0.66	66.00
30	PM375	17	94.11%	0.92	86.58
31	PM478	7	100.00%	0.83	83.00
32	TC9H09	11	90.90%	0.88	80.00
33	TC3E05	4	0.00%	0.75	00.00
34	TC3D04	8	75.00%	0.87	65.25
35	TC2C03	1	0.00%	0.00	00.00
	Mean	4.82	64.95%	0.62	44.91

Table 5. Summary of the AMOVA within and among groundnut genotypes.

Source	df	SS	MS	Est. Var.	%	Stat	Value	P value
Among Pops	1	8.271	8.271	0.700	15%			
Among Indiv.	11	43.575	3.961	3.961	85%	phipt	0.150	0.025
Total	12	51.846		4.662	100%			

Table 6. Association of SSR markers with HKM based on Kruskal-Wallis one way ANOVA.

Sr. No.	Primers	Hc	R2
1	TC3A12	20.82 (0.0200)	0.282
2	TC4G02	49.07 (0.0015)	0.010
3	TC9B08	41.28 (0.0035)	0.027
4	PM478	41.66 (0.0046)	0.082
5	TC9H09	68.98 (0.0008)	0.121
6	TC3D04	57.87(0.0444)	0.074

Values mentioned in parenthesis indicate p value

Table 7. Genotypes with diverse background tested for validation of associated locus.

Sr. No	Variety	HKM (g)	Kernel size
1	BAU13	59.40	Large
2	TKG19A	54.21	Large
3	NRCGCS 148	56.59	Large
4	TPG41	56.00	Large
5	CHICO	14.65	Small

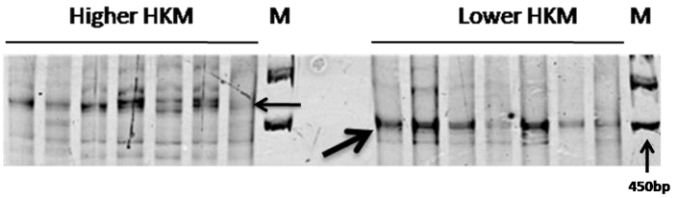


Fig 4a. TC3A12 locus amplified in higher HKM mutants and absent in lower HKM mutants. Arrows indicate amplification of specific bands to genotypes with higher HKM and lower HKM in addition to 450bp DNA ladder.

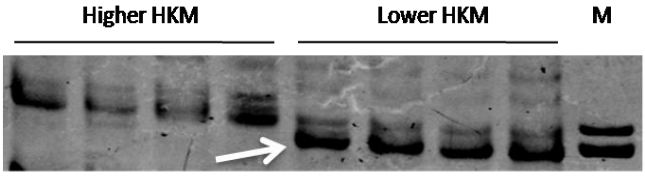


Fig 4b. Amplification of TC3A12 locus in selected mutant genotypes with higher HKM and lower HKM. Arrow indicates the amplifications of a specific band in mutant genotypes with lower HKM which is 450bp in size and absent in mutant genotypes with higher HKM.

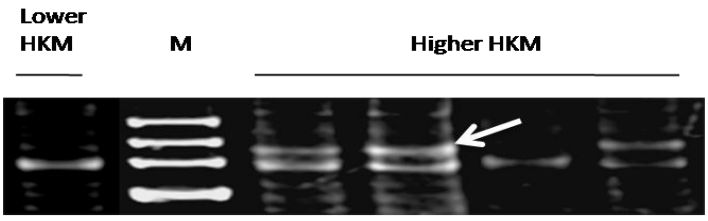


Fig 5. TC3A12 locus amplified in three out of four varieties with higher HKM and was absent in the variety with lower HKM. Arrow shows the amplified band associated with HKM.

Fig 6. Higher HKM mutant without pod constriction identified from NRCGCS-281**.**

(Genei, Banglore India), 5µl Mili-Q water, 0.5µl each of forward and reverse primers (25 pmoles). PCR amplification was performed in C1000 thermal cycler (BIO-RAD USA). Thirty cycles of 30 seconds at 94°C for denaturation of template, 1 minute at 54°C for primer annealing followed by 30 seconds at 72°C for primer extension. The DNA was size separated by horizontal electrophoresis in 2% agarose (Lonza, Rockland, ME, USA) and stained in ethidium bromide (0.1%). The resolved amplification products were scanned using laser scanner (Fujifilm FLA 5100, Japan). The amplification products were scored as 1 (presence) and 0 (absence) across the lanes comparing their respective sizes. Only strong, reproducible and clearly distinguished bands were used in the following analysis.

Statistical analysis

Polymorphism per cent was estimated using following formaula. Polymorphism %= (number of polymorphic bands/total number of bands in that assay unit) x 100.

PIC was determined using following formula as described by Powell et al., 1996. PIC = $[1-\sum fi^2]$, Where f is the frequency of it allele averaged across loci.

Marker index (MI) was calculated by applying following formula given by Powell et al., 1996 and Smith et al., 1997.
MI = polymorphism (%) x PIC value.
BSA analysis was done by pooling separately the DNA samples of genotypes with higher HKM together and genotypes with lower HKM together.
Genetic similarity analyses were performed using SIMQUAL program in NTSYS (Rohlf, 2000). Cluster analysis was performed using UPGMA based on Jaccard's similarity coefficient. AMOVA and regression co-efficient were calculated using GenAlEx 6 (Peakall and Smouse, 2006) software and Kruskal-Wallis was calculated using PAST version 2.07 software (Hammer et al., 2001).

Conclusion

In this study average polymorphism and PIC of SSRs observed were 64.95% and 0.62, respectively. Cluster analysis revealed 61% Jaccard's similarity coefficient among genotypes and confirmed common pedigree of genotypes. AMOVA detected 15% of total variation associated with kernel mass and K-W ANOVA detected significant association between SSRs with kernel mass. Two SSRs namely TC3A12 and TC9H09 were associated with major QTLs for HKM in groundnut.

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