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Identification of stable sources for surrogate traits in *Arachis glabrata* and marker-trait association for tolerance to water deficit stress

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Abstract: Diploid and tetraploid wild relatives of cultivated peanut are genetically diverse, providing rich sources of genetic resources for tapping resistance to various biotic and abiotic stresses. *Arachis glabrata* Benth. is a tetraploid (2n = 40) rhizomatous species that is reported to be tolerant to water deficit stress. Identification of *A. glabrata* accessions with high SPAD chlorophyll meter reading (SCMR) and low specific leaf area (SLA) values would be an option for improving water deficit stress tolerance in peanut. An investigation was undertaken to study the genetic diversity of *A. glabrata* accessions using molecular markers as well as surrogate traits (SLA and SCMR) for water deficit stress to identify stable sources for SCMR and to identify DNA markers linked to SCMR. SCMR showed high heritability and genetic advance as percent of mean (GAM) and a negative relation with SLA in *A. glabrata*. High heritability and high GAM indicates additive gene action. A high level of genetic diversity was observed in *A. glabrata* accessions, indicating that these populations had not experienced major genetic bottlenecks or genetic drift. Eight random amplified polymorphic DNA markers were identified with significant association to SCMR.

Key words: *Arachis*, analysis of molecular variance, association, genetic diversity, SPAD chlorophyll meter reading, specific leaf area

1. Introduction

Peanut (*Arachis hypogaea* L.) is an important oilseed crop grown in 24 × 10^6 ha throughout the world (FAOSTAT, 2010). It is mainly grown in semiarid tropical regions under rainfed conditions. Water deficit stress is one of the major abiotic stresses that have depressive effects on peanut productivity (Nageswara Rao et al., 1989; Nautiyal et al., 2002; Nigam et al., 2005), causing a yield loss of US$ 500 million every year (Sharma and Lavanya, 2002).

Specific leaf area (SLA) and SPAD chlorophyll meter reading (SCMR) have been reported as important surrogate traits for tolerance to drought in peanut. Studies by Wright et al. (1994) observed that SLA was closely and negatively correlated with water use efficiency (WUE). SLA is significantly influenced by factors such as time of sampling and leaf age (Wright and Hammer, 1994; Nageswara Rao et al., 1995), suggesting the need for further studies on the factors influencing WUE in peanut (Nageswara Rao et al., 2001). Nageswara Rao et al. (2001) reported a significant and high interrelationship among SLA and SCMR in peanut, suggesting that SCMR could be used as a reliable and rapid measure to identify genotypes with low SLA and high WUE.

Extensive variation for SLA and other leaf characters exists in wild *Arachis* L. species (Nautiyal et al., 2008) with abundant DNA polymorphisms (Kochert et al., 1991; Halward et al., 1993; Garcia et al., 1996; Burow et al., 2001; Garcia et al., 2005; Moretzsohn et al., 2013) suggesting the need for molecular characterization of wild peanut germplasm for SLA and SCMR (surrogate traits for WUE).

Wild species have proven their importance in improvement of biotic and abiotic stresses in many crops (Simpson et al., 2003; Fernie et al., 2006; Tanksley and Fulton, 2007; Fu et al., 2010; Nevo and Chen, 2010). Likewise, wild *Arachis* species exhibit many desirable traits related to disease, pest and insect resistance, and drought tolerance. On the other hand, the cultivated peanut has been characterized by a narrow genetic base (Halward et al., 1991, 1992; Raina et al., 2001; Gimenes et al., 2002; Herselman, 2003; He et al., 2005). Thus, introgression breeding would certainly help in widening the narrow genetic base of cultivated peanut. It is, therefore, critical to determine the levels of genetic diversity available in wild relatives for SCMR and SLA and identify desirable donors to broaden the genetic base of the crop (Singh et al., 1997). *Arachis glabrata* Benth. was reported to thrive well under water deficit stress conditions (Nautiyal et

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al., 2008) and remained largely unexploited. *Arachis glabrata* is highly cross-incompatible with *A. hypogaea*. However, Mallikarjuna and Sastri (1985a, 1985b, 2002) and Mallikarjuna (2002) reported fertile hybrids between *A. hypogaea* and *A. glabrata* and the transfer of genes for biotic and abiotic stresses to *A. hypogaea*.

Introgression of desirable traits could be more efficient and successful with the use of molecular markers and genetic linkage maps (Mohan et al., 1997; Samizadeh et al., 2003; Varshney et al., 2005; Burow et al., 2008; Selvaraj et al., 2009; Varshney et al., 2009; Khedikar et al., 2010; Mondal and Badigannavar, 2010; Ravi et al., 2010; Gautami et al., 2011; Shirasawa et al., 2012; Sujay et al., 2012). In the recent past, good progress was made for tagging a few economically important traits (Mace et al., 2006; Selvaraj et al., 2009; Mondal et al., 2012) and genetic linkage maps have been developed using both wild species (Burow et al., 2001; Moretzsohn et al., 2005) and cultivars in peanut (Varshney et al., 2009; Sujay et al., 2012).

Success in marker-assisted selection (MAS) depends on accurate phenotyping of the breeding lines, which is difficult sometimes in natural field conditions and/or in simulated artificial conditions. Climatic conditions vary not only with locations and growing seasons, but also from year to year at the same location. The phenotype reflects nongenetic as well as genetic influence on plant growth and development. Effects of genotype and environment are not independent (Comstock and Moll, 1963). Knowledge on the performance and/or adaptability of genotypes to particular environments is highly important to estimate the agronomical value of the genotype and allows recommendations for specific environments. The selection of genotypes, based on the stability in different environments rather than on trait means in a specific environment, is a strategy to reduce the genotype × environment interaction (Eberhart and Russell, 1966). Thus, a stable wild accession identified for a trait of interest would unravel variation for many useful traits not observed in the cultivated species.

In the present study, we report 1) genetic diversity of *Arachis glabrata* accessions using DNA markers [simple sequence repeat (SSR) and random amplified polymorphic DNA (RAPD)], 2) genetic variability of *A. glabrata* accessions based on surrogate traits (SLA and SCMR) for tolerance to drought, 3) stable accessions for SCMR, and 4) association of markers with SCMR in *A. glabrata* accessions.

2. Materials and methods

In this study, 34 *Arachis glabrata* accessions of various origins have been used (Table 1). Stem cuttings of perennial *A. glabrata* accessions were collected from the International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India. Cuttings were propagated and maintained in closed-bottom concrete round pots of 66 cm in diameter and 46 cm in height filled with a mixture of soil and sand at a 1:1 ratio under field condition (Figure 1). Fertilizers (DAP and urea) were applied at 6-month intervals at a ratio of 25:60:30 (N:P:K) for luxurious growth. Pots were irrigated on alternate days except in the rainy season (July to September).

2.1. Surrogate traits for water deficit stress

In each accession, 30 leaves from the 2nd and 3rd positions from the top of the main axis or branches were collected from 5 randomly selected plants per replication to record SCMR and SLA values, using the equation SLA = leaf area (cm^2) / leaf dry weight (g). Leaf area was measured using a leaf area meter (LI 3000 LI-COR, USA) followed by drying at 80 °C in a hot air oven for leaf dry weight. SCMR was recorded as described by Nigam and Aruna (2008) using a SPAD meter (SPAD-502, Minolta Corp., USA) from the leaves collected for recording SLA.

2.2. Isolation of genomic DNA

Genomic DNA was extracted from tender leaf samples collected from field-grown plants following a CTAB method (Saghai-Maroof et al., 1984). The quality of total DNA isolated was checked in NanoDrop spectrophotometer (Model ND 1000, NanoDrop Technologies, USA) at an A_{260}/A_{280} ratio as well as in 2% agarose gels.

2.3. Molecular markers

RAPD (Table 2) SSR primers (Table 3) obtained from Integrated DNA Technologies (USA) were used in the study. The polymerase chain reaction (PCR) mixture (8 µL) contained 0.5 µL (50 ng) of genomic DNA, 2.5 U of Taq DNA polymerase, 0.75 µL of 10X Taq buffer (Genei, India), 0.5 µL of dNTPs (10 mM) (Genei), 5 µL of Milli-Q water, and 0.5 µL each of forward and reverse primers (25

Figure 1. *Arachis glabrata* accessions maintained in concrete pots under field conditions.

Table 1. Details of 34 *Arachis glabrata* accessions used in the present study.

Gen no.	NRCG no.	ICG no.	Org	Lat (°S)	Long (°W)	Elev (m)
G1	11841	8177	PRY	23.25	57.5	100
G2	11837	8173	PRY	23.25	57.5	100
G3	11835	8171	BRA	18.28	54.46	247
G4	11833	8169	BRA	20.43	54.33	450
G5	11839	8175	PRY	23.25	57.5	100
G6	11828	8161	PRY	22.13	56.53	700
G7	11823	8156	PRY	27.32	55.78	80
G8	11824	8157	PRY	27.32	55.78	80
G9	11816	8149	BRA	20.26	55.22	225
G10	11831	8166	BRA	22.57	55.13	400
G11	11830	8165	BRA	22.07	56.48	250
G12	11846	8188	BRA	-	-	-
G13	11832	8167	BRA	21.5	54.3	400
G14	11815	8148	BRA	-	-	-
G15	11821	8154	ARG	44.3	65.35	-
G16	11819	8152	BRA	21.43	57.26	200
G17	11817	8150	BRA	20.26	55.23	225
G18	11818	8151	BRA	20.5	55.53	250
G19	11813	8145	ARG	-	-	-
G20	11847	8902	BRA	-	-	-
G21	11834	8170	BRA	20.5	54.24	570
G22	11838	8174	PRY	23.25	57.50	100
G23	11826	8159	PRY	25.29	56.35	220
G24	11820	8153	BRA	21.39	57.19	200
G25	11822	8155	PRY	27.32	55.78	80
G26	11825	8158	PRY	25.25	56.58	220
G27	11836	8172	BRA	-	-	-
G28	11844	8184	BRA	-	-	-
G29	11845	8185	BRA	-	-	-
G30	11829	8162	BRA	22.7	56.32	200
G31	11842	8179	BRA	-	-	-
G32	12036	8950	BRA	18.55	54.33	300
G33	12033	8921	ARG	27.45	57.36	60
G34	11840	8176	PRY	23.25	57.50	100

Abbreviations: Gen no. = Genotype number, Org = origin, Lat = latitude, Long = longitude, Elev = Elevation, PRY = Paraguay, BRA = Brazil, ARG = Argentina.

Table 2. RAPD primers used in the amplification of *Arachis glabrata* accessions with their sequence and annealing temperature (T_M).

No.	Primer no.	Sequence	T_M (°C)
1	D5	5'- TGAGCGGACA -3'	37.1
2	D6	5'- ACCTGAACGG -3'	34.3
3	D7	5'- TTGGCACGGG -3'	40.9
4	D9	5'- CTCTGGAGAC -3'	29.5
5	OPT5	5'- GGGTTTGGCA -3'	35.8
6	OPT6	5'- CAAGGGCAGA -3'	34.1
7	OPT7	5'- GGCAGGCTGT -3'	40.3
8	OPI5	5'- TGTTCCACGG -3'	34.9
9	OPI6	5'- AAGGCGGCAG -3'	41.1
10	OPI7	5'- CAGCGACAAG -3'	33.5

Table 3. SSR primers used in the amplification of *Arachis glabrata* accessions with their sequence and annealing temperature (T_M).

Primer		Sequence	T_M (°C)
SSR			
PM15	F	5'- CCTTTTCTAACACATTCACAC ATGA -3	53.7
	R	5'- GGCTCCCTTCGATGATGAC -3	55.4
PM402	F	5'- CCGCCCTAAAAACTGTATTCG -3	53.9
	R	5'- CCTAAGAGTACACGCGACGA -3	56.2
PM375	F	5'- CGGCAACAGTTTTGATGGTT -3	54.2
	R	5'- GAAAAATATGCCGCCGTTG -3	52.7
PM32	F	5'-AGTGTTGGGTGTGAAAGTGG GGGACT -3	63.9
	R	5'- CGGAACAGTGTTTATC -3	43.9
PM188	F	5'- GGGCTTCACTGCTTTTGATT -3	53.8
	R	5'- TGCGACTTCTGAGAGGACAA -3	55.8
PMc588	F	5'- CCATTTTGGACCCCTCAAAT -3	53.1
	R	5'- TGAGCAATAGTGACCTTGCATT -3	54.7
PMc478	F	5'- GTCGTGCAGGTCAAAGTGC -3	57.0
	R	5'- TTAAGATGGGTGCCTGCAAT -3	54.6
PMc297	F	5'- ATGCACCTGCAAGTGAAGAG -3	55.5
	R	5'- TCAAGGATGCAGCAAGACAC -3	55.5
TC1A02	F	5'- GCAATTTGCACATTATCCGA -3	51.6
	R	5'- CATGTTCGGTTTCAAGTCTCAA -3	53.4
TC11A04	F	5'- ACTCTGCATGGATGGCTACAG -3	56.9
	R	5'- CATGTTCGGTTTCAAGTCTCAA -3	53.4
TC7C06	F	5'- GGCAGGGGAATAAAACTACTA ACT -3	54.5
	R	5'- TTTTCCTTCCTTCTCCTTTGTC -3	52.7
TC2D06	F	5'- AGGGGGAGTCAAAGGAAAGA -3	55.6
	R	5'- TCACGATCCCTTCTCCTTCA -3	55.2
TC1E01	F	5'- CAGCAAAGAGTCGTCAGTCG -3	55.8
	R	5'- GAAAGTTCACTTGAGCAAATTCA -3	52.1
TC9F10	F	5'- ATCACAATCACAGCTCCAACAA -3	54.9
	R	5'- GGCAAGTCTAATCTCCTTTCCA -3	54.5
TC11E04	F	5'- ACGACACCCTGAAATCAAGTTT -3	54.8
	R	5'- CCGAAGGCACCAAAAAGTAT -3	53.2

pmol). PCR amplification was performed in a C 10000 thermal cycler (Bio-Rad, USA). For RAPD primers, samples were held at 94 °C for 4 minutes for complete denaturation of the template DNA, followed by 35 cycles of 1 min at 94 °C for denaturation of the template, 30 s at 37 °C for primer annealing, and 1 min at 72 °C for primer extension. For amplification with SSRs, 44 cycles of 30 s at 94 °C for denaturation of the template, 1 min at 54 °C for primer annealing, and 30 s at 72 °C for primer extension were used.

The DNA was size-separated by horizontal electrophoresis in 3% agarose gels (Lonza, USA) and stained in ethidium bromide (0.1%). The resolved amplification products were scanned using a laser scanner (Fujifilm FLA 5100, Japan). The amplification products were scored as 1 (presence) or 0 (absence) across the lanes comparing their respective sizes. Only strong, reproducible, and clearly distinguishable bands were used in the following analysis.

2.4. Statistical analysis

The level of polymorphism was measured by the number of polymorphic bands using the following equation: polymorphism % = number of polymorphic bands / total number of bands in that assay unit. Polymorphic information content (PIC) values in this study were calculated by the following algorithm: PIC [or the diversity index of Nei (1973)] = $1 - \sum f i^2$, where f is the frequency of the ith allele averaged across loci.

Marker index (MI) value was calculated for the RAPD and SSR markers by applying the formula given by Powell et al. (1996) and Smith et al. (1997): MI = polymorphism (%) × PIC value.

Analysis of variance (ANOVA) was performed to check significance of genotypes between treatments. Variance components for SCMR and SLA were calculated by partitioning phenotypic variance into genotypic variance, environmental variance, and genotypic × environmental interaction variance. Furthermore, Finlay–Wilkinson stability analysis (Finlay and Wilkinson, 1963) was done for SCMR over 3 environments, the 2006 post-rainy season (February to May), 2006 rainy season (June to October), and 2007 post-rainy season (February to May). The model was presented with the following equation:

$$F_{ij} = \mu_{ij} + g_{ij} + b_i t_j + \delta_{ij} + e_{ij},$$

where μ_{ij} is the average yield of the ith genotype in the jth environment, g_i is the average yield of the ith genotype in all environments, b_i is the regression coefficient to the environmental index indicating a genotypic response to environmental changes, t_j is an environmental index as a mean of all genotypes in the jth environment reduced by a grand mean, and δ_{ij} is the deviation from regression of the ith genotype in the jth environment.

Heritability in the broad sense was computed for the 2 characters by the formula suggested by Lush (1949). The predicted genetic advance (in the broad sense) was estimated according to the formula given by Johnson et al. (1955) using PBSTAT v.1.2 (Suwarno et al., 2008). Principal component analysis was performed for molecular data and significant principal components obtained were used to construct a cluster diagram by Ward's method (Ward, 1963). A general similarity coefficient matrix was developed as suggested by Gower (1971) using PAST software (Hammer et al., 2001).

Accessions were grouped into 'low-SCMR' and 'high-SCMR' groups based on SCMR values. Accessions having SCMR values greater than the mean of 34 accessions formed the high-SCMR group, whereas accessions having SCMR values lower than the mean formed the low-SCMR group. Analysis of molecular variance (AMOVA) was performed using GenAlex (Peakall and Smouse, 2006) to study significant variance between the 2 groups. Regression analysis was used to detect associations between marker (genotypic) classes (presence or absence of the band) and their respective phenotypic values. The data on each marker were subjected to nonparametric Kruskal–Wallis one-way analysis of variance using PAST software (Hammer et al., 2001) to identify markers potentially linked to SCMR.

3. Results

SSR markers have been found most successful in identifying molecular variation within the cultivated peanut species (Hopkins et al. 1999; Ferguson et al., 2004a, 2004b; Mace et al., 2006, 2007; Cuc et al., 2008; Varshney et al., 2009; Khedikar et al., 2010; Mondal and Badigannavar, 2010; Gautami et al., 2011; Shirasawa, 2012; Sujay et al., 2012). However, a few RAPD markers have been successfully used in the identification of candidate genes of interest in peanut (Burow et al., 1996; Garcia et al., 1996; Mondal et al., 2007).

In this study, 34 *Arachis glabrata* accessions of different origins were analyzed for genetic diversity using 10 RAPD and 15 SSR primers. The 10 RAPD primers yielded 140 bands, of which 132 were polymorphic, with an average of 13.2 polymorphic fragments per primer (Table 4). The number of amplified fragments per primer ranged from 11 to 18 and the size of amplicons ranged from 50 to 1500 bp. Among these primers, OPT6 produced the maximum number of bands (18), while the lowest number of bands (11) was produced by OPT5. Polymorphism ranged from 72.7% to 100.0%, with an average of 73.3%. All RAPD primers were highly polymorphic (≥50%). The PIC values for RAPD primers were higher than 0.90, with an average value of 0.92. The MI value of RAPD primers varied from 65.4 (OPT7) to 93.9 (D7), with a mean value of 86.2. The RAPD primers used in the study had high polymorphism, PIC values, and MI values.

Table 4. Amplification of *Arachis glabrata* accessions by RAPD and SSR primers.

No.	Primer	G+C (%)	No. of bands			Polymorphism (%)	PIC	MI
			M	P	Total			
RAPD								
1	D5	60.0	0	13	13	100.00	0.91	91.28
2	D6	60.0	0	14	14	100.00	0.93	92.69
3	D7	70.0	0	17	17	100.00	0.94	93.88
4	D9	60.0	0	13	13	100.00	0.92	91.75
5	OPT5	60.0	1	10	11	90.91	0.90	81.99
6	OPT6	60.0	1	17	18	94.44	0.94	88.62
7	OPT7	70.0	2	12	14	85.71	0.92	78.96
8	OPI5	60.0	1	12	13	92.31	0.92	84.55
9	OPI6	70.0	0	16	16	100.00	0.93	93.10
10	OPI7	60.0	3	8	11	72.73	0.90	65.35
	Total		8	132	140			
	Mean		0.8	13.2	14.0	73.31	0.92	86.22
SSR								
1	PM15	43.1	0	6	6	100	0.82	82.41
2	PM402	51.2	1	7	8	87.5	0.84	73.59
3	PM375	46.1	0	10	10	100	0.88	88.42
4	PM32	42.8	0	7	7	100	0.82	82.41
5	PM188	47.5	0	3	3	100	0.64	64.43
6	PMc588	42.8	0	8	8	100	0.86	85.56
7	PMc478	51.2	0	3	3	100	0.52	52.14
8	PMc297	50.0	0	5	5	100	0.79	78.76
9	TC1A02	40.4	0	2	2	100	0.39	38.56
10	TC11A04	46.5	0	6	6	100	0.78	77.70
11	TC7C06	39.1	0	2	2	100	0.49	49.38
12	TC2D06	50.0	0	9	9	100	0.88	88.35
13	TC1E01	41.8	0	9	9	100	0.88	88.03
14	TC9F10	43.1	0	5	5	100	0.78	77.72
15	TC11E04	43.9	0	6	6	100	0.79	78.75
	Total		1	88	89			
	Mean		0.07	5.87	5.93	99.17	0.748	74.73

M = Monomorphic, P = polymorphic.

Molecular analysis of *Arachis glabrata* accessions using 15 SSR primer pairs yielded 89 bands, of which 88 were polymorphic, with an average of 5.9 polymorphic fragments per primer pair. The number of amplified fragments ranged from 2 to 10 per primer pair. Among these primers, PM375 produced the maximum number of bands (10), while the lowest number of bands (2) was produced by both TC1A02 and TC7C06. All the primers except PM402 showed 100% polymorphism. The PIC of 15 SSRs varied from 0.4 (TC1A02) to 0.9 (PM375) with a mean value of 0.74. The MI of SSRs varied from 38.6 (TC1A02) to 88.4 (PM375) with a mean value of 74.7 (Table 4). Out of 15 SSRs, 8 had high polymorphism, PIC values, MI values, and number of alleles amplified.

Ward's (1963) method of clustering using RAPD and SSR markers in the present study discriminated the 34 *Arachis glabrata* accessions into 6 clusters (Figure 2). The maximum number of accessions (10) were grouped into cluster 2, while the minimum number of genotypes (3) were grouped into cluster 4 and cluster 5. Similar grouping of accessions was observed by principal component analysis with minor deviation (Figure 3). The grouping of accessions was neither consistent with the country of origin nor with their elevations (Table 1). Accessions G7, G8, and G25, suspected as being duplicates, grouped into separate clusters (ICRISAT, 2012).

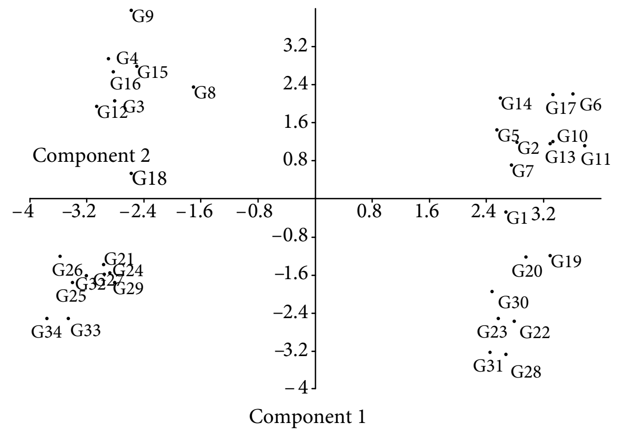

Figure 3. PCA scatter diagram of PC1 vs. PC2 depicting distribution of 34 *Arachis glabrata* accessions.

Dissimilarity coefficients obtained using Gower's similarity coefficient among the accessions of *Arachis glabrata* ranged from 0.16 to 0.57 (Table 5). Highest genetic similarity was observed between G6 and G 7 and between G7 and G8. Lowest genetic similarity was observed between accessions G16 and G34, which differed in their countries of origin.

ANOVA for SLA and SCMR in 34 *Arachis glabrata* accessions over 3 seasons had significant variability for

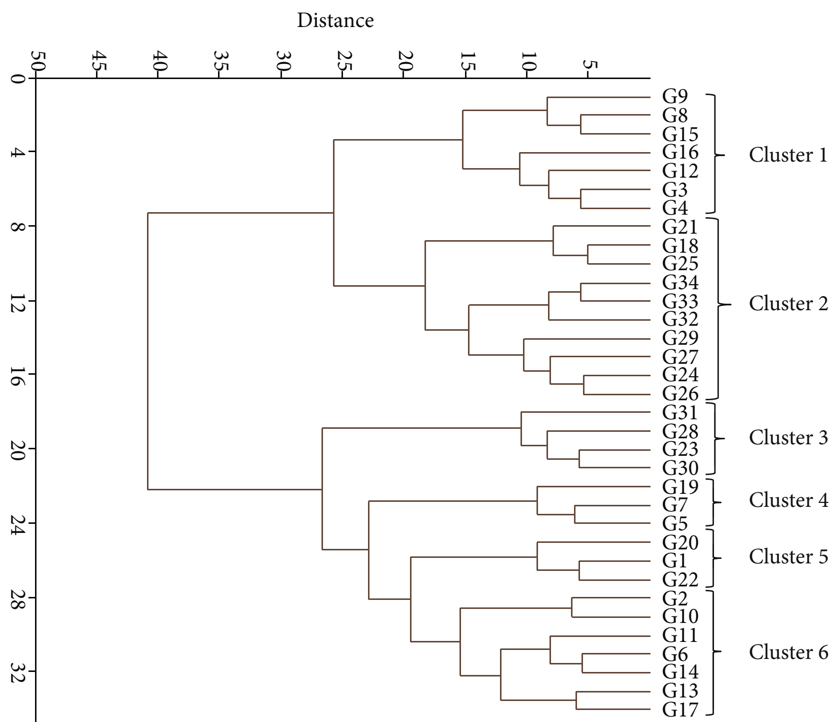

Figure 2. Dendrogram constructed with Ward's method of clustering with 34 *Arachis glabrata* accessions using 229 binary data (RAPD and SSR).

Table 5. Pairwise matrix of Gower's genetic distance among *Arachis glabrata* accessions estimated by RAPD and SSR markers.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
1	0.00																																
2	0.23	0.00																															
3	0.27	0.25	0.00																														
4	0.25	0.22	0.20	0.00																													
5	0.26	0.24	0.21	0.21	0.00																												
6	0.21	0.27	0.26	0.22	0.25	0.00																											
7	0.20	0.22	0.22	0.23	0.23	0.16	0.00																										
8	0.26	0.26	0.24	0.26	0.18	0.21	0.16	0.00																									
9	0.33	0.26	0.24	0.23	0.27	0.26	0.27	0.26	0.00																								
10	0.30	0.30	0.29	0.30	0.24	0.29	0.28	0.26	0.31	0.00																							
11	0.24	0.30	0.28	0.27	0.25	0.22	0.28	0.29	0.30	0.33	0.00																						
12	0.24	0.24	0.24	0.20	0.26	0.24	0.24	0.25	0.25	0.30	0.24	0.00																					
13	0.32	0.28	0.24	0.26	0.26	0.29	0.30	0.33	0.30	0.35	0.28	0.27	0.00																				
14	0.26	0.27	0.21	0.22	0.24	0.21	0.24	0.25	0.23	0.29	0.25	0.19	0.23	0.00																			
15	0.29	0.29	0.27	0.29	0.30	0.24	0.24	0.24	0.26	0.28	0.31	0.29	0.27	0.21	0.00																		
16	0.38	0.34	0.30	0.33	0.34	0.34	0.35	0.36	0.28	0.37	0.30	0.30	0.31	0.27	0.29	0.00																	
17	0.31	0.30	0.28	0.26	0.28	0.26	0.29	0.28	0.27	0.33	0.28	0.25	0.25	0.26	0.22	0.24	0.00																
18	0.35	0.41	0.45	0.40	0.45	0.41	0.38	0.45	0.48	0.46	0.44	0.41	0.47	0.41	0.41	0.50	0.45	0.00															
19	0.47	0.49	0.46	0.44	0.50	0.47	0.41	0.49	0.51	0.47	0.48	0.45	0.50	0.46	0.47	0.52	0.48	0.22	0.00														
20	0.44	0.45	0.49	0.49	0.50	0.44	0.46	0.51	0.55	0.52	0.49	0.47	0.52	0.45	0.45	0.52	0.46	0.19	0.26	0.00													
21	0.44	0.48	0.50	0.47	0.49	0.44	0.45	0.49	0.50	0.52	0.43	0.47	0.53	0.42	0.43	0.51	0.46	0.19	0.26	0.21	0.00												
22	0.38	0.48	0.53	0.48	0.52	0.48	0.46	0.51	0.53	0.49	0.45	0.46	0.49	0.45	0.47	0.53	0.44	0.24	0.26	0.22	0.20	0.00											
23	0.48	0.48	0.48	0.45	0.50	0.47	0.45	0.48	0.50	0.52	0.46	0.45	0.45	0.44	0.44	0.49	0.45	0.25	0.26	0.25	0.16	0.20	0.00										
24	0.44	0.45	0.42	0.43	0.46	0.43	0.43	0.49	0.48	0.50	0.45	0.42	0.42	0.40	0.45	0.47	0.42	0.25	0.24	0.22	0.21	0.21	0.21	0.00									
25	0.39	0.41	0.47	0.44	0.48	0.47	0.43	0.47	0.49	0.49	0.44	0.40	0.48	0.43	0.45	0.47	0.48	0.20	0.26	0.21	0.23	0.20	0.22	0.17	0.00								
26	0.48	0.45	0.48	0.45	0.52	0.46	0.42	0.48	0.46	0.48	0.49	0.45	0.46	0.44	0.44	0.48	0.41	0.28	0.27	0.24	0.27	0.23	0.23	0.17	0.20	0.00							
27	0.47	0.51	0.48	0.47	0.49	0.46	0.40	0.47	0.50	0.49	0.47	0.44	0.48	0.48	0.48	0.50	0.46	0.24	0.26	0.28	0.24	0.23	0.23	0.22	0.20	0.22	0.00						
28	0.50	0.48	0.50	0.49	0.51	0.53	0.47	0.51	0.52	0.48	0.53	0.48	0.48	0.45	0.47	0.44	0.47	0.26	0.27	0.29	0.27	0.27	0.24	0.26	0.24	0.24	0.23	0.00					
29	0.43	0.48	0.48	0.49	0.48	0.48	0.43	0.49	0.54	0.49	0.45	0.49	0.44	0.43	0.41	0.47	0.45	0.24	0.31	0.29	0.26	0.24	0.27	0.24	0.23	0.25	0.24	0.18	0.00				
30	0.49	0.50	0.44	0.43	0.46	0.47	0.43	0.43	0.47	0.47	0.44	0.42	0.48	0.43	0.43	0.45	0.42	0.25	0.26	0.29	0.26	0.25	0.20	0.25	0.21	0.25	0.20	0.19	0.22	0.00			
31	0.45	0.51	0.48	0.50	0.52	0.50	0.46	0.49	0.52	0.52	0.45	0.45	0.52	0.47	0.48	0.48	0.48	0.31	0.28	0.29	0.30	0.27	0.26	0.27	0.22	0.26	0.23	0.23	0.21	0.21	0.00		
32	0.47	0.48	0.46	0.51	0.50	0.49	0.47	0.46	0.55	0.50	0.48	0.44	0.50	0.47	0.49	0.49	0.48	0.29	0.28	0.29	0.31	0.29	0.32	0.24	0.20	0.27	0.24	0.24	0.25	0.23	0.21	0.00	
33	0.48	0.48	0.48	0.50	0.50	0.47	0.44	0.53	0.54	0.48	0.46	0.45	0.45	0.47	0.47	0.52	0.46	0.26	0.27	0.26	0.24	0.29	0.23	0.20	0.24	0.23	0.24	0.24	0.22	0.24	0.25	0.23	0.00
34	0.52	0.51	0.50	0.53	0.54	0.55	0.51	0.54	0.55	0.50	0.50	0.48	0.49	0.50	0.52	0.57	0.51	0.32	0.33	0.32	0.31	0.25	0.28	0.22	0.23	0.21	0.26	0.30	0.28	0.25	0.27	0.24	0.19

genotype, environment, and genotype × environment interaction (Table 6). The components of phenotypic variance (V_P) due to genotype (V_G) and genotype × environment interaction ($V_{G \times E}$) are presented in Table 7. The magnitude of genotypic variance was higher (11.9) than genotype × environment interactions (6.7) in SCMR. However, the magnitude of genotype × environment interactions (460.6) was higher than genotypic variance (231.5) in the case of SLA. Heritability estimates in a broad sense for SCMR and SLA were 83.2% and 54.0%, respectively. In addition, genetic advances as percent mean of (GAM) for SCMR and SLA were 23.2 and 8.4, respectively. Thus, SCMR in *A. glabrata* showed higher heritability as well as higher GAM in comparison with SLA.

Furthermore, Finlay–Wilkinson stability analysis (Finlay and Wilkinson, 1963) was done for SCMR over 3 environments, the 2006 post-rainy season (February to May), the 2006 rainy season (June to October), and the 2007 post-rainy season (February to May), among 34 *Arachis glabrata* accessions to identify stable genotypes (Table 8). Regression coefficients ranged from 0.13 (G31) to 2.24 (G8). Thirteen genotypes had regression coefficients greater than 1.00, while 4 genotypes (G3, G19, G23, and G26) had regression coefficients close to unit (bi = 0.95 to 1.1) and the remaining 17 genotypes had coefficients of less than 1.0. The mean squared deviation from the regression coefficient (S^2di) ranged from 0.02 to 5.28. The regression model defines a stable variety as having an above-average mean (Pi), a regression coefficient of unity (bi = 1.0), and a nonsignificant mean square for deviations from regression ($S^2di = 0$). None of the genotypes satisfied this criteria; however, G30 had an above-average mean (Pi > 0), bi close to 1.00, and low S^2di, and hence its performance may not change with a change in environmental conditions. Genotypes G10, G11, G20, and G28 had Pi > 0, bi > 0, and low S^2di, and hence are sensitive to environmental changes. Genotypes G1, G6, G13, G22, and G31 had Pi > 0, low S^2di, and bi less than 1.0. Performance of these genotypes will not change irrespective of environmental conditions and they may not respond to conducive environments, either. Genotypes G2 and G4 have steady performances under poor environments (Pi < 0, low bi, and low S^2di) and may not respond to conducive environments.

AMOVA between Low-SCMR and High-SCMR groups indicated a significant difference between (8%) and within (92%) groups (Table 9). Markers associated with SCMR based on single-marker analysis using regression and the nonparametric methods of marker association

Table 6. Analysis of variance for SLA and SCMR among *Arachis glabrata* accessions.

Source	df	SCMR		SLA		
		MS	F-count	MS	F-count	1%
Season	2	610.7	526.0**	30,319.7	287.7**	4.83
Replication	3	1.2	1.0^{ns}	105.4	0.4^{ns}	3.99
Genotype	33	86.1	5.9**	2574.6	2.2**	1.86
G × S*	66	14.5	12.9**	1185.5	4.5**	1.67
Error	99	1.1		264.4		
Total	203					
CV %		3.38			10.03	

*: G × S = Genotype × environment interaction, **: statistically significant, ns: not significant.

Table 7. Estimation of phenotypic variance parameters and broad sense heritability among *Arachis glabrata* accessions for SLA and SCMR.

Source	V_G	$V_{G \times E}$	V_P	h^2_{bs} (%)	GA	GAM
SCMR	11.9	6.7	14.3	83.2	6.5	23.2
SLA	231.5	460.6	429.1	54.0	23.0	8.4

V_G = Genotypic variance, V_P = phenotypic variance, $V_{G \times E}$ = genotypic × season interaction variance.

Table 8. Finlay–Wilkinson stability analysis for SCMR among 34 *Arachis glabrata* accessions.

Var.	Mean	Pi	bi	R^2	S^2di
G1	35.4	4.0	0.2	0.3	1.4
G2	28.3	–3.2	0.3	0.2	2.3
G3	30.8	–0.7	1.0	1.0	0.2
G4	30.3	–1.1	0.3	1.0	0.1
G5	29.5	–1.9	0.4	0.1	4.5
G6	37.4	6.0	0.4	0.1	5.3
G7	26.8	–4.6	1.6	1.0	0.2
G8	30.3	–1.1	2.2	1.0	0.0
G9	31.1	–0.3	0.2	0.1	2.5
G10	34.2	2.8	1.9	1.0	1.3
G11	35.5	4.1	1.6	1.0	0.9
G12	31.3	–0.1	0.4	0.1	4.8
G13	31.8	0.4	0.2	0.6	0.6
G14	31.2	–0.2	0.9	0.7	2.6
G15	27.0	–4.4	2.0	1.0	1.5
G16	31.1	–0.3	1.7	1.0	1.6
G17	31.8	0.4	0.8	0.8	1.9
G18	27.2	–4.2	1.8	0.9	2.6
G19	33.2	1.8	1.0	0.9	1.1
G20	33.0	1.6	1.3	1.0	1.0
G21	30.8	–0.6	2.0	1.0	1.4
G22	32.4	1.0	0.3	0.3	2.1
G23	31.6	0.2	1.1	0.8	2.2
G24	30.1	–1.3	1.4	1.0	0.3
G25	29.8	–1.6	1.2	0.5	4.9
G26	32.5	1.1	1.0	0.9	1.1
G27	31.4	0.0	0.8	0.8	1.8
G28	37.3	5.9	2.2	1.0	0.0
G29	35.0	3.6	0.8	0.9	1.2
G30	42.1	10.7	1.2	1.0	0.6
G31	33.1	1.7	0.1	0.0	5.0
G32	24.6	–6.8	0.7	0.7	2.0
G33	28.0	–3.4	0.9	0.5	3.7
G34	22.1	–9.3	0.9	0.5	3.8

Table 9. AMOVA for 34 genotypes in 4 clusters obtained by Ward's method employing 10 RAPD and 15 SSR markers.

Source	df	SS	MS	Est. var.	Variance	Stat	Value	P
Among pops	1	123.3	123.3	4.4	8%			
Within pops	32	1551.1	48.5	48.5	92%			
Total	33	1674.4		52.9	100%	PhiPt	0.083	0.01

Kruskal–Wallis test H: 1098, $P < 0.0001$.

are presented in Table 10. The Kruskal–Wallis test was performed in order to obtain an estimate of each locus contribution to the differentiation between 2 SCMR groups and compared with R^2 values. The Kruskal–Wallis test demonstrated that they were associated with the SCMR (P-value ranged from 0.04 to 0.008). Phenotypic variation as accounted by these markers ranged from 14.8% to 33.4%.

Table 10. List of alleles putatively linked with SCMR as determined through locus-by-locus AMOVA % differentiation between 'High-SCMR' and 'Low –SCMR' groups among 34 *Arachis glabrata* accessions.

Locus	Allele	He	P-value	AMOVA % diff.
D5	1	11.8	0.003	33.4
	2	6.4	0.04	17.1
	3	6.4	0.04	29.9
	4	6.4	0.04	22.9
	5	11.8	0.003	33.4
D6	1	9.3	0.008	27.3
D9	1	6.4	0.04	23.2
	2	9.3	0.008	23.2
	3	7.2	0.04	14.8
OPT5	1	8.8	0.02	33.2
	2	9.3	0.008	23.2
OPT6	1	11.9	0.008	32.8
	2	6.4	0.04	28.6
	3	9.8	0.008	18.0
	4	9.3	0.008	23.2
OPT7	1	9.3	0.008	23.2
	1	5.7	0.04	17.2
	2	9.3	0.008	23.2
	3	9.3	0.008	23.2
	4	9.3	0.008	23.2
OPI5	1	9.8	0.008	22.9
OPI6	1	9.3	0.008	18.7

He = Expected heterozygosity.

4. Discussion

DNA fingerprinting using molecular markers is a routine method employed to study the extent of genetic diversity across a set of germplasms or cultivars as well as to identify desired parents and for MAS in different crop species (Cooke, 1995; Collard et al., 2005; Mukherjee et al., 2013; Muthiah et al., 2013).

RAPD, a dominant PCR-based marker, has been reported as less polymorphic in cultivated peanut (Subramanian et al., 2000; Mondal et al., 2005). However, with limited sequence information in peanut, a few economically important traits, namely nematode resistance (Burow et al., 1996) and rust resistance (Mondal et al., 2007), have been tagged using RAPD markers. In contrast, SSRs are codominant PCR-based markers and more polymorphic in cultivated peanut than RAPDs. In the recent past, good progress was made in the development of SSRs as well as in tagging of economically important genes in peanut (Selvaraj et al., 2009; Varshney et al., 2009; Hong et al., 2010; Mondal and Badgannavar, 2010; Gautami et al., 2011; Ravi et al., 2011; Mondal et al., 2012; Qin et al., 2012; Sujay et al., 2012).

SSR primer pairs used in this study amplified 4 to 18 alleles. Some SSR primer pairs amplified more than one locus, which could be due to loci duplication in *Arachis glabrata*, a tetraploid species. Meiotic analysis of *A. glabrata* produced tetravalents (2.3 per cell), univalents (1.1 per cell), and occasional trivalents (0.2 per cell), although having 20 bivalents in a cell was not an uncommon feature (Mallikarjuna, 2004). Amplification of more than one fragment by primer pairs/markers in peanut was reported in earlier studies (Hopkins et al., 1999; Krishna et al., 2004; Kottapalli et al., 2007; Varshney et al., 2009) and has been attributed to either amplification of duplicated loci or different loci, because of the tetraploid genome. Liang et al. (2009) observed 3–18 alleles in wild species, which was attributed mainly to differences in repeat type and length in the microsatellite regions.

Accessions with unique alleles may be useful for introgressing diversity into cultivated peanut, which has a narrow genetic base, for crop improvement. Further evaluation of these novel alleles may provide some association with useful traits for peanut breeders. SSR markers used in this study were highly polymorphic and efficient in revealing the level of genetic diversity present in the tetraploid accessions studied.

High levels of genetic diversity in *Arachis glabrata* accessions indicated that these populations had not experienced any major genetic drift so far, since *A. glabrata* is solely asexually propagated through rhizomes and characterized with cleistogamous flowers. Natural cross-pollination occurs at rates of less than 1% to greater than 6% due to atypical flowers or action of bees (Coffelt, 1989). Hence, *A. glabrata* is maintaining its genetic variations as such without much gene flow. In nature, the amount of genetic diversity found in plant populations is often a function of the rate of gene flow (Bruschi et al., 2003).

In this study, *Arachis glabrata* accessions belonging to different countries of origin were clustered together by multivariate analytical tools, which may be attributed to: 1) high levels of polymorphism detected at the analyzed loci; 2) occurrence of homoplastic alleles, i.e. alleles that present the same size (bp) in a gel but are not identical by descent (Varshney et al., 2005); and 3) allele-sharing between accessions belonging to different genomes. In addition, *A. glabrata* accessions used in these studies were found to differ in SLA as well as SCMR, 2 surrogate traits of WUE in peanut (Wright et al., 1996; Nageswara Rao et al., 2001; Bindu Madhava et al., 2003; Chunilal et al., 2006; Nigam and Aruna, 2008). Environment (season) as well as genotype × environment interactions had significant influence on the expression of SLA and SCMR. This indicates that the expression of these 2 traits in *A. glabrata* is influenced by the environment. The magnitude of genotypic variance was greater than genotype × environment interactions in SCMR in contrast to SLA, indicating that expression of SCMR is more genetically controlled while expression of SLA is more influenced by the changes in environment. In addition, SCMR had high heritability and high GAM in both environments. On the other hand, SLA had high heritability as well as high GAM in post-rainy seasons, while, in the rainy season, it had moderate heritability with high GAM.

In the present study, variance due to genotype × environment interactions for SCMR was lower than SLA along with high heritability, indicating that SCMR was a more stable parameter than SLA in *Arachis glabrata* and confirming the earlier observation made by Upadhyaya (2005) in a mini core collection of peanut. Nigam and Aruna (2008) also reported that variance due to genotypes × time of observation was very small for SCMR in cultivated groundnut. High heritability coupled with high genetic advance irrespective of seasons indicated additive gene action for SCMR, and thus selection in early generations could be more effective than for SLA. In contrast, Babitha et al. (2006) observed high heritability and low GAM in F_2 generations of 5 out of 6 peanut crosses, predicting that SCMR may be controlled by many genes under the influence of nonadditive gene action.

Success of any crop breeding program depends on availability of stable parents. The interactions between genotype and environment can influence the selection process and recommendation of undesirable parents vis-à-vis failure of crop breeding programs. Stability is defined as the ability of a certain variety to maintain stable yield under changing environmental conditions (Yılmaz and

Tugay, 1999). Thus, stability analysis of 34 *Arachis glabrata* accessions based on SCMR value, a stable surrogate of WUE, using the Finlay and Wilkinson (1963) model, resulted in identification of stable reliable parents for improving WUE in peanut. Finlay and Wilkinson (1963) defined that bi ~ 1.00 indicates average stability, bi < 1.00 below average stability, and bi > 1.0 above average stability. However, varietal adaptability must be determined based on size of bi in unison with mean. Accordingly, varieties with bi > 1.00 and genotypic mean higher than grand mean (Pi > 0) possess below-average stability. They are highly sensitive to environment. Thus, under most favorable environment, such varieties may produce maximum results. Varieties with bi < 1.00 and Pi > 0 possess above-average stability, i.e. low sensitivity to environment. They yield average results under poor conditions but low in rich environments. Accordingly, accessions G1, G6, G13, G17, G22, and G31 would be preferred under poor (water deficit stress) environments because these accessions would be less sensitive to environmental changes and produce average performance. Accessions G10, G11, G20, and G28 would be better choices under favorable environment and small changes in environmental conditions would greatly affect their SCMR values. In contrast, accessions G19, G23, and G26 exhibit average stability and are suitable to all environments, and changes in environment would have little or no effect on their performances. Accessions identified for stable expression will make good parents for improving SCMR vis-à-vis tolerance to water stress in peanut.

AMOVA has indicated that only 8% of total variation is accounted for between low and high SCMR groups, whereas the majority of variation was accounted for (92%) within the SCMR groups. The diversity analysis reported in the present study was also used to identify loci linked to SCMR for use in MAS. Sun et al. (2003) studied diversity among 35 spring wheat genotypes with different levels of resistance to *Fusarium* head blight using 160 RAPD markers and were successful in identifying 3 RAPD markers associated with FHB resistance. This indicates that unrelated parents could be used to identify markers linked to agronomically important traits, and such markers can be used as candidate markers for further gene mapping. They further highlighted that this approach is advantageous over bi-parental populations as the markers identified are likely to be applicable to large breeding program. In this study, all RAPD primers were highly polymorphic and 8 primers were found to be associated with SCMR through the Kruskal–Wallis test and regression analysis. This study has identified 4 markers, $D5_{-1}$, $D5_{-5}$, $OPT5_{-1}$, and $OPT6_{-1}$, linked to SCMR with more than 30% phenotypic variance.

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