Characterization of Simple Sequence Repeat (SSR) Markers and Genetic Relationships within Cultivated Peanut (*Arachis hypogaea* L.)

Yan Li[1], Charles Y. Chen[2], Steve J. Knapp[3], Albert K. Culbreath[1], C. Corley Holbrook[4], and Baozhu Guo[5]*

ABSTRACT

A total of 709 SSR markers were collected from public databases and 556 SSRs passed an initial screen and were used to characterize 16 peanut (*Arachis hypogaea*) genotypes. PIC (polymorphism information content) scores and heterozygosity indices for each marker were calculated to assess the genetic diversity revealed by SSR markers and genetic distances were estimated from shared allele distances for construction of a cladogram by the Neighbor-Joining method to illustrate the genetic relationships among the genotypes. Two hundred thirty-five (42.27%) markers showed polymorphisms in these genotypes. The average heterozygosity estimated from these 556 SSRs was 0.225 with a range of 0 to 0.992 and the average PIC was 0.209. The average number of alleles per SSR was 2.5 with a range of 1 to 13. However, 410 SSR markers had only one allele, confirming that diversity of cultivated peanuts is very limited. Among the polymorphic SSR markers, 26.4% were dinucleotide GA repeat motif markers, followed by dinucleotide CT (10.4%), and trinucleotide TAA (9.6%). The dinucleotide and trinucleotide repeat motifs are the most abundant type of SSRs, and dinucleotide GA repeat motif shows a higher polymorphism in comparison to other types. The genetic relationships revealed from the cladogram are in agreement with the pedigrees and origins of the tested peanut genotypes, indicating that these SSR markers are useful tools for evaluation of genetic diversity in peanuts.

Key Words: SSRs, polymorphism, genetic relationship, cultivated peanut.

Peanut (*Arachis hypogaea* L.) probably originated in southern Bolivia or northern Argentina in South America (Gregory *et al.*, 1980; Kochert *et al.*, 1996). The genus *Arachis* contains approximately 70 species (Krapovickas and Gregory, 1994), and almost all of them are diploid with either an A genome or a B genome. In contrast, the cultivated peanut species is a tetraploid (2n = 4x = 40 chromosomes) composed of both the A and B genomes.

In the past, morphological similarity was widely used for estimating variation within species (Ayana and Bekele, 1999), and variations in morphological, physiological, and agronomic traits have also been reported in peanuts. Recently, development of DNA molecular markers, such as RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism), and SSR (simple sequence repeat), have been used to assess genetic variability and conduct evolutionary studies in different crops (Burr and Burr, 1991; Akkaya *et al.*, 1992; Rongwen *et al.*, 1995; Cho *et al.*, 2000; Temnykh *et al.*, 2000; Gethi *et al.*, 2002; Robertson-Hoyt *et al.*, 2006). There are many advantages of using these molecular markers including high repeatability, no genotype × environment (G × E) interaction, and no off season limitation. In addition to assessing genetic variability, these markers also can be very useful for characterization of individuals and breeding lines for choosing parental genotypes in breeding programs (Ribaut and Hoisington, 1998). Previous research has indicated a low level of genetic variability in cultivated peanut (Halward *et al.*, 1991, 1992; Kochert *et al.*, 1991; Stalker *et al.*, 1994; He and Prakash, 1997; Gupta and Varshney, 2000). Compared to other kinds of markers such as RFLPs and RAPDs, SSR markers are co-dominant and multi-allelic in inheritance, have higher level of DNA polymorphism in cultivated peanut, and are easier to amplify with less DNA quantity and low cost (Hopkins *et al.*, 1999; Tang *et al.*, 2003; He *et al.*, 2003). The identification and characterization of molecular markers in cultivated peanut has lagged behind other economically important crops such as soybean, maize, and rice because of the limited genetic variability in cultivated peanut, and the added complexity of having two genomes.

In this research, the majority of SSR polymorphic markers detected by different research groups were collected and screened with sixteen cultivated peanut genotypes having a range of field reactions to Tomato spotted wilt virus (TSWV), *Cercospora*

[1]University of Georgia, Department of Plant Pathology, Tifton, GA 31793.
[2]USDA-ARS, National Peanut Research Laboratory, Dawson, GA 39842.
[3]University of Georgia, Department of Crop and Soil Sciences, Athens, GA 30602.
[4]USDA-ARS, Crop Genetics and Breeding Research Unit, Tifton, GA 31793.
[5]USDA-ARS, Crop Protection and Management Research Unit, Tifton, GA 31793.
*Corresponding author (e-mail: Baozhu.Guo@ars.usda.gov)

Table 1. Sixteen peanut genotypes used for evaluation of SSR markers.

Genotype	Origin	Market Type
Tifton 8	U.S. germplasm	Virginia
C724-19-25	U.S. breeding line	Runner
Georgia Green	U.S. cultivar	Runner
Georganic	U.S. cultivar	Runner
Spancross	U.S. cultivar	Spanish
Tifguard	U.S. cultivar	Runner
NC-6	U.S. cultivar	Virginia
SunOleic 97R	U.S. cultivar	Runner
Tifrunner	U.S. cultivar	Runner
UF NC 94022-1-2-1-1-b3-B	U.S. breeding line	Runner
PE-2	Chinese breeding line	Virginia
PE-1	Chinese breeding line	Virginia
GTC-20	Chinese cultivar	Spanish
GTC-9	Chinese cultivar	Spanish
Tennessee Red	U.S. cultivar	Valencia
GP-NC WS13	U.S. germplasm	Virginia

arachidicola (early leaf spot) and *Cercosporidium personatum* (late leaf spot). The objectives of this study were: 1) to characterize the SSR markers based on allele-length ranges, average PIC (polymorphism information content) score and heterozygosity among 16 genotypes of cultivated peanut; 2) to detect the genetic relationships among these 16 genotypes; and 3) to explore potential genotypes for use as parents for recombinant inbred line (RIL) population development for genetic mapping.

Materials and Methods

Plant materials

A diverse array of 16 genotypes tested in this study was obtained from USDA-ARS and the University of Georgia peanut programs at the University of Georgia Coastal Plain Experiment Station in Tifton, GA in 2006 (Table 1). These genotypes included commercial cultivars as well as breeding lines and germplasm accessions from the U.S. and China. Some of the lines have been previously evaluated for resistance to TSWV and leaf spot pathogens, and wide ranges of variation for resistance to TSWV, *C. arachidicola* and *C. personatum* have been observed. 'Georgia Green' (Branch, 1996) was a predominant cultivar planted in Georgia with a moderate level of field resistance to TSWV and has been used as a standard moderate resistance cultivar to TSWV (Culbreath *et al.*, 1999, 2008). 'Georganic' (tested previously as C11-2-39) (Holbrook and Culbreath, 2008) and 'Georgia-01R' (Branch, 2002) are cultivars with resistance to TSWV and moderate levels of resistance to the early and late leaf spot pathogens (Holbrook *et al.*, 2008a, 2008b). 'Tifguard' (tested previously as C724-19-15) and a near-isogenic sister line 'C724-19-25' also have field resistance to TSWV but differ in susceptibility to the peanut root knot nematode (Holbrook *et al.*, 2008b). 'Tifrunner' (Holbrook and Culbreath, 2007) was released in 2007 as a new cultivar with field resistance to TSWV and moderate resistance to early and late leaf spots. 'SunOleic 97R' (Gorbet and Knauft, 2000) was a cultivar developed by the University of Florida with good agronomic traits including high oleic acid oil composition (>80%), but it is susceptible to TSWV (Culbreath *et al.*, 2005) and leaf spot pathogens. The breeding line 'F NC94022-1-2-1-1-b3-B' (hence forth referred to as 'NC94022') has been reported to have a high level of field resistance to TSWV(Culbreath *et al.*, 2005).

DNA extraction

Leaves of 16 genotypes were collected from greenhouse grown plants at Tifton, GA for total DNA extraction. The fresh leaf tissues were frozen at −80°C after harvesting, and then ground in liquid nitrogen with a mortar and pestle. The total genomic DNA was extracted according to a modified CTAB method (Tang *et al.*, 2002) from fresh leaves. DNA concentration and quality were determined by use of a spectrophotometer at 260 nm wavelength and a ratio of 260nm/280nm. Afterwards, DNA was diluted in sterile water to 10 ng/ul for PCR reaction.

SSR markers

A total of 709 pairs of SSR markers were collected and used for screening polymorphisms in this study (Table 2). Among them, 97 pairs of primers were newly developed from genomic sequences in Dr. Steve Knapp's Laboratory at the University of Georgia (Table 3). An additional 612 pairs of primers were chosen from 6 different research groups' published data. Initially, SSR markers were screened on 1.5% agarose gels for utility, functionality, and length estimation using the bulk DNA of 4 randomly chosen cultivated peanut samples. From the initial screen, 153 SSR markers were deleted with no amplification. Therefore, only 556 SSR markers passed the initial screen and were used to assess polymorphisms in the 16 genotypes. These SSR primers were synthesized by MWG Biotech (High Point, N.C., USA). Forward primers were modified by adding different fluorescent phosphoramidite (6FAM, HEX, or TAMRA) to the 5′ ends. Two hundred thirty-seven primers were labeled with 6-FAM (6-carboxyfluorescein), 236 primers were labeled with HEX (hexachloro-carboxyfluorescein), and the remaining 236 primers were labeled with TAMRA (tetramethyl rhodamine) (Tang *et al.*, 2002, 2003).

Table 2. Sources of 709 SSR markers used for initial screening for functional markers.

Markers	Number of Markers Designed	Markers yielding amplification[1]	Reference
GM1-GM271	271	186 (68.6%)	Moretzsohn *et al.*, 2005
GM272-GM338	67	56 (83.6%)	Moretzsohn *et al.*, 2004
GM339-GM344	6	6 (100%)	Hopkins *et al.*, 1999
GM345,GM346	2	1 (50%)	Krishna *et al.*, 2004
GM347-GM364	7	4 (57.1%)	Palmieri *et al.*, 2002
	11	4 (36.4%)	Palmieri *et al.*, 2005
GM365-GM420	56	47 (83.9%)	He *et al.*, 2003
GM421-GM612	192	158 (82.3%)	Ferguson *et al.*, 2004
GM613-GM709	97[2]	94 (96.9%)	Knapp, unpublished
Total	709	556 (78.4%)	

[1]Number of functional markers were used in the initial screening. Numbers in parenthesis are percentage of functional markers.
[2]These 97 new SSR markers are listed in Table 3.

PCR amplification

'Touchdown' PCR (Don *et al.* 1991) was used to minimize spurious amplification. An 11.5 ul reaction volume in 384-well plates was used for PCR reaction, containing 1 ul of forward and reverse primers, 2 ul of DNA template, 1.15 ul of 10 × PCR buffer, 0.25 ul of dNTP (2.5 mM) , and 0.1 ul of *Taq* enzyme. The 'touchdown' thermal cycle of the PCR reaction was decided by the thermal temperature (Tm) for different kinds of primers separately and was applied with either 52°C or 56°C 'touchdown' cycle reaction. The 'touchdown' amplification program is as follows: 94°C for 1 min to allow samples to denature, followed by 6 cycles of 94°C for 30s, 62°C for 30s, and 72°C for 30s, the annealing temperatures were decreased 1°C per cycle in subsequent cycles until the temperature reached 52°C or 56°C for the different kinds of 'touch down' programs. Products were subsequently amplified for 36 cycles at 94°C for 20s, 56°C for 20 s, and 72°C for 30 s with a final extension for 20 min.

Electrophoresis and detection of fluorescent products by multiplex

PCR-multiplexes were used based on the multiplex PCR principles described by Edwards and Gibbs (1994) and Henegariu *et al.* (1997), and performed as described by Tang and Knapp (2003) using post-PCR multiplexing of six amplicons (samples were diluted 60- to 100-fold). The criteria used to select SSR markers for the PCR-multiplexes were primer compatibility, genotyping performance when amplified by multiplex PCR, and allele length range (estimated from the SSR polymorphism initial screen). The SSR markers were sorted by allele-length range and combined so as to minimize the comigration of identically labelled non-allelic bands.

The PCR products were first checked with 1.5% agarose gel to ensure the successful amplifications, and then were diluted 60- to 100- fold before analysis using an ABI3730. Those six different diluted amplicons with different fluorescent labels and amplification length were mixed into one well by 1 uL with 9 ul of formamide containing a GeneScan 500 internal lane standard labeled with ROX. GeneScan Filter Set D and the ROX 500 internal-standard were used for analyses of amplicons labeled with FAM, HEX, and TAMARA. Gene Mapper 4.0 was used for allele scoring for the 556 functional SSR markers based on the multiplex mixture with different fluorescent labels and amplification lengths.

Statistical analysis

The screening results for the 556 markers were recorded as 1 for presence of the amplification band, 0 for absence of the amplification band. According to the amplification quality and reliability across 16 genotypes, the markers were classified as 1–5, where 1= Excellent, indicated by perfect amplification with unambiguous product within all 16 genotypes; 2 = Good, indicated by clear peak with some magnification; 3 = Fair, indicated by clear peak with high magnification and 1–2 genotypes with null amplification results; 4 = Poor, indicated by bad amplification results and 2–4 genotypes with null amplification results; 5 = Not good, indicated that more than half genotypes had null amplification results. Failed PCR amplifications were scored as missing data for genotyping.

The PIC (polymorphism information content) value, defined by Botstein *et al.* (1980) as a closely related diversity measure is a measure of the polymorphism of a marker (SSR marker) for linkage. The formula for this estimation of PIC score is:

$$\hat{PIC}_l = 1 - \sum_{u=1}^{k} \tilde{p}_{lu}{}^2 - \sum_{u=1}^{k-1} \sum_{v=u+1}^{k} 2\tilde{p}_{lu}{}^2 \tilde{p}_{lv}{}^2$$

Where l = index for marker 'l'; $\tilde{p}_{lu}$ = proportion of marker 'l' alleles which are of allele type 'u'; $\tilde{p}_{lv}$ =

Table 3. Newly developed SSR markers from methylation-filtered and shotgun genomic sequences for diploid and tetraploid peanut taxa.

Marker	SSR Marker	Forward (5′-3′)	Reverse (5′-3′)	Ta	Product Length (bp)	Repeat Motif	GenBank Accession Number
GM613	GA1	gcgtgaaatgagtgttgtgag	catagccaccatagacaccaaa	60	394	(AT)21	DX511190
GM614	GA2	gtgtcctgtggtgtccaaatta	tgcataccggagaaattcaag	60	444	(CAC)5+(CAA)6	DX511218
GM615	GA4	ctcaccaaggaatggttgaga	cgccaaaatgcatataagagc	60	201	(AATG)5	DX511282
GM616	GA5	aaacttggacgttggctttgt	ttgatccagaacctgaaagga	60	176	(ATT)6	DX511305
GM617	GA6	cctcttcttgatttcgtgctg	gaaacatacattcctcttgcatca	60	489	(TTA)6	DX511395
GM618	GA8	tgaagggaggaagagacgaat	cctctgtgcgacatgactctaa	60	230	(GA)19	DX511449
GM619	GA12	aaacgagaagcgttgagttagc	cgttgaagaagaagctcgaaa	60	227	(CGC)7	DX511610
GM620	GA14	tgaagtcaccaagcattctcc	tgatccagaacctgaaaggac	60	155	(ATT)6	DX511738
GM621	GA19	accaacgatgctgctgataac	atcctcaacccatgctttctt	60	494	(AT)9	DX514046
GM622	GA20	acacccataacacaccacaagt	accgaagcacagagaagacaa	60	337	(ACAT)5	DX514055
GM623	GA21	caggatgaacaggcacagaat	atgaacaattgcgatttggac	60	365	(TAT)16	DX514093
GM624	GA24	aacgaaatattttgagaaaggat	agcattagcaactctaagctcat	60	203	(TAT)22	DX514187
GM625	GA26	cctcacttctttttgcatggt	tggaaaggaaatgatttggtg	60	164	(AT)15	DX514254
GM626	GA27	catccaaagccaaagttcaca	gcttagcttgctttgattaggg	60	200	(GA)31	DX514344
GM627	GA28	agatggtggtgtaggagttgtgt	tggccgttggatatttatttg	60	177	(TC)9	DX514345
GM628	GA29	cttttcccagatgcattttga	cccaaattgagattgaccaaa	60	148	(AATA)8	DX514379
GM629	GA30	caagagggacggataatagca	gacgcaaggaaatgagcatac	60	393	(TTA)6	DX514445
GM630	GA32	cagcaattcagcaaactaatgaa	tcctcccacgtccttttattt	60	341	(ATA)11	DX514493
GM631	GA33	caagtgatagcacgctgtttg	ttaagtcccatgcctgtcttg	60	471	(AT)13	DX514496
GM632	GA34	ttcaatcatttcacgtgtcaatc	agtgctaggagccagcaattt	60	309	(TC)5(TA)9	DX514509
GM633	GA35	caaagtttgcagtgattttgttg	aaattttcaggtaaatcattctt	60	208	(AAT)18	DX514517
GM634	GA37	cctcacaaaggctaaagaggaa	tataccatccctgccacagaa	60	323	(AT)26	DX514526
GM635	GA42	ttgtgcgaagggtaagatagaaa	tcctgtgcttgaatctggaat	60	191	(TC)11	DX514692
GM636	GA43	ggccagaacaaatcggttatt	ttgttcaagatttagtcactcaacata	60	197	(AAT)6	DX514698
GM637	GA44	tgactttaattttgagcttcctataa	ttttctgtccataattatatcgtattt	60	288	(AT)10	DX514723
GM638	GA45	agcacattggcgagatcacta	agcaaactggagagaagcaca	60	412	(GGC)7	DX514725
GM639	GA47	taggtgcctttgcttctcttg	ccatctcttgtctttgttcatgg	60	299	(TGTA)5	DX514818
GM640	GA48	aatggcactgtgaaggacaac	ttccggttatggagcatctac	60	296	(AATG)5	DX514864
GM641	GA49	acgtttccccaataagaccac	tggacaccttatcggcttatc	60	493	(AT)20	DX514897
GM642	GA50	cgaaatcgcatatatcctcga	taacaatagccacggactcga	60	248	(TGT)6	DX514917
GM643	GA51	aggaagtggcttcaagggtat	ctgtctcctgaaagttgggttt	60	275	(TATC)8	DX514933
GM644	GA53	tctatgttcccggtgtacgtt	taatgcctcacttggagttgg	60	291	(TGT)6	DX514985
GM645	GA54	gccatcaggagaagaacttga	tgctgagagagagagcaatgag	60	302	(GGA)6+(GAAG)5	DX515017
GM646	GA56	tttggtctctctcctccatca	tttccagcatcaatagcaacc	60	246	(TC)11+(CT)7	DX515121
GM647	GA57	atcgtcctcgcaggttctta	cttgatttggtaatgggctga	60	292	(AT)29	DX515131
GM648	GA58	aaattcacattcatctaaccatttca	gagtgctagaggcagcaacttt	60	369	(AAT)5	DX515157
GM649	GA59	ccctctgctccatattcatca	ggtagtggtggtgcatgtttt	60	457	(CCA)7+(ACC)4	DX515160
GM650	GA60	gaggaagagcaagccaagaat	ctcgcaccaattacacaaacc	60	488	(CGG)6	DX507857
GM651	GA61	ctaccttcggcatggtgatt	tcgagcccttctttgactct	60	343	(TGT)9	DX507915
GM652	GA62	tccaaaatacacattctcatgg	ctacacgcaaaaagctcagaa	60	184	(AG)26	DX507921

(Table 3 continued)

Table 3. Continued

Marker	SSR Marker	Forward (5′-3′)	Reverse (5′-3′)	Ta	Product Length (bp)	Repeat Motif	GenBank Accession Number
GM653	GA64	gagtggtcttttggtctgtgc	tgtttggactttgcatgttttc	60	294	(ATTA)7	DX507927
GM654	GA65	ttgcatgttagtcccaccact	ccgcactcgtttggaaatta	60	400	(GA)9	DX508003
GM655	GA68	gaagactattgcaccctccaa	cggcactgaatagagcaatgt	60	434	(CT)10+(CA)7	DX508204
GM656	GA71	gccacaacaataacaacacca	ggcataatcgaacaaatgcag	60	213	(CAC)8	DX508326
GM657	GA72	actttggtggctttccttcat	tctctgtgccctctttcttca	60	388	(ATA)36	DX508347
GM658	GA73	gcaacaccattaacgatcacc	tccacgttccttcttccctaa	60	221	(TTA)8	DX508398
GM659	GA79	cgcaatgagaaattcaacagc	tgataattcttccgcgattgt	60	127	(AGAA)9	DX508693
GM660	GA80	tgaaagtaactcgtttacagtttgaag	tcactaaacatgtgggtaactaagaaa	60	300	(TG)14	DX508758
GM661	GA81	aaggaacggcgtaatagcatc	tatctaaccctagccgccatt	60	334	(TTTC)5	DX508765
GM662	GA84	ttgcagaacactccaacatcc	tttaggtggtggcggtgaat	60	323	(GCC)8	DX508884
GM663	GA85	gcatattgcatggtaacgaag	ggggatgtgtattaatttacttga	60	173	(TA)10	DX508927
GM664	GA87	cttcacctccaaaatcaacca	accgctgacatttgattgttc	60	302	(CCA)6	DX512002
GM665	GA88	atttgaacggcagagagcata	tcattccctccctcacatctt	60	306	(ACC)6	DX512035
GM666	GA91	tgaactttgggagtgtatatgacc	cccacacccctaaattatgt	60	467	(TTA)10	DX512194
GM667	GA96	accaagtcaaatgttgcccta	cgttcttcctctgttccttca	60	338	(TA)19	DX512271
GM668	GA99	aaaatgcccctatcccttctt	tgattttctgagtttggcagtg	60	101	(CCA)6	DX512414
GM669	GA101	tgaaatgatgcaaccacacat	aagggaaagtaaaaccatgcaa	60	343	(TA)16	DX512441
GM670	GA102	agagtctcgtgctaataacgtgt	tggataacccattttctatgttg	60	379	(AT)24	DX512462
GM671	GA108	tggatgctgtaaggaatggac	ttatcgagcttgcctcagaaa	60	297	(ATT)6	DX512922
GM672	GA110	ggagaaccagtgacgtgacata	ggattaattctgataccatgaaagg	60	243	(CT)11	DX513012
GM673	GA119	cgatgctctcttccttcctct	ccgctactccctaactcaagc	60	125	(TTTC)5	DX513795
GM674	GA120	ctaaccctagccgccatacc	gttgatggcttcacggtgag	60	460	(AAAG)6+(GA)9	DX513812
GM675	GA122	cccatctcaagtgtaaaagtgct	cctccccacccttaaacaata	60	254	(TA)9	DX513856
GM676	GA124	gcggaaacagaaacaaatcaa	gccgttacataccagaggaaa	60	315	(CT)9	DX513942
GM677	GA127	ggcgcagaagttacgaagagt	tttcattcaataacgtacatccat	60	282	(TA)23	DX515321
GM678	GA131	atgtccttgccttgtttcgtt	tagttggcggtatggcttaga	60	176	(GT)7+(GA)10+(GT)7	DX515409
GM679	GA133	ggtgttatgtatagccaccag	aaatagtatggaccagaaataataag	60	433	(ATA)20	DX515570
GM680	GA135	cagaagctgaagttgaaccaga	caccttgtttcgtcgtttgtt	60	494	(ATC)6	DX515619
GM681	GA138	tccagtgagggttaatagctcaa	agagtgaaggtagaatggtggaa	60	302	(TA)16	DX515880
GM682	GA140	ttaggctggtggaaagtgatg	caaataaaacaatgaattgataatcg	60	246	(TAA)6	DX515900
GM683	GA141	aacatctctctcccacccaat	cagggatctcttgatttgcag	60	285	(TCT)6	DX515903
GM684	GA145	tctcacttccatcgttgctct	aagcgtggcgttacaagtatg	60	240	(ATG)7	DX515986
GM685	GA146	ggcttaaactccgcaagctac	tcacacgaaactaacgcacaa	60	148	(AT)23	DX516011
GM686	GA147	ctcagcatagcaacacacacg	ggcttctccttcccagtcct	60	266	(GA)12	DX516063
GM687	GA150	tccacaatgtgtagttcaagca	tggtgaccatttcaaactcttg	60	479	(AT)12	DX516119
GM688	GA151	atcaatgcacatgggacaaa	gggctgaggtaaaggtaagca	60	261	(TA)34	DX516137
GM689	GA155	atcgtgcagaggaatagcaga	gcgtagttaatttgcgaggtg	60	456	(TA)31	DX516215
GM690	GA156	ctactccctctgctgcttcct	tagggtttcgttgaggaggtt	60	208	(CT)26	DX516252
GM691	GA160	tctttatcccgatgaatgaaa	ctcccacaaacacaaacacac	60	334	(GA)22	DX516350
GM692	GA161	tgaggccgtcttgtttagaga	cctcttccatcaccgttcata	60	196	(GA)35	DX516432

(Table 3 continued)

Table 3. Continued

Marker	SSR Marker	Forward (5′-3′)	Reverse (5′-3′)	Ta	Product Length (bp)	Repeat Motif	GenBank Accession Number
GM693	GA163	atgtatagtggcggatccaat	tttgaagtattctcttttcaaca	60	486	(TA)12	DX516592
GM694	GA165	atttgtgccctaccaccttct	tccctcctagaggttgacttga	60	386	(TA)11+(GA)28	DX516642
GM695	GA166	tggtcgcagatagtatttctcct	tggaatttgaatcgcactctt	60	437	(ATA)19	DX516680
GM696	GA167	ccttagatggcaacattggaa	acctaaaccctaacacccacac	60	280	(CTTT)5	DX516729
GM697	GA168	cttgaatggttctacgcttcg	gattatcttcgttcatttccatca	60	125	(TGA)6	DX516788
GM698	GA169	ctccttaacaataaatcgagtgatga	caacccatttgccacctctat	60	434	(AT)30	DX516814
GM699	GA172	cataccgacagatttgaactcg	gttactgttgctgccaccatt	60	418	(AAT)14	DX517092
GM700	GA174	agctttcctgatttgccactt	gccaaacaagtttagaacaatcc	60	336	(CT)9	DX517187
GM701	GA175	catccaacacttccaaatacca	ggtaagcatgaagaacgcaaa	60	271	(TTAG)5	DX517272
GM702	GA177	gataggccataaacatgtcc	tgagggttgagctgaagagaa	60	235	(TCT)6	DX517327
GM703	GA194	ttgggttttgagagtgagaa	ccttgaccttcctttgcctta	60	194	(AAAT)5	DX509824
GM704	GA195	aaacatgctcctgcctctct	tcagagtacaatcatgcaatcaaa	60	478	(TCC)5	DX509886
GM705	GA196	gtctgaggacatgacagaacca	cgtaccactctccaccatgc	60	264	(CGA)6	DX509899
GM706	GA197	tggaagaaaatggaaggaaca	ggggattccaatcattcacat	60	278	(AGA)6	DX509961
GM707	GA198	actggcattatggtagtaggataaca	cattgcgctgcacaacttac	60	498	(AAGA)5+(GA)5	DX509992
GM708	GA199	tgctttgtttatgtacctcttgga	caatctgtccctcaggcttta	60	481	(ATT)19	DX510089
GM709	GA217	acagacagcgcagattagagg	aaggaacattaaacctggatcg	60	196	(CT)9	DX511001

proportion of marker 'l' alleles which are of allele type 'v'; k = number of alleles types present for marker 'l' (Shete *et al.*, 2000). Another estimator, heterozygosities (H) were estimated for each SSR marker as described by Ott (1991) according to the formula:

$$H = 1 - \sum_{i=1}^{n} p_i^2$$

Where P*i* is the frequency of i^{th} allele in the genotype population (Shete *et al.*, 2000). The estimation of PIC, amplified allele number, allelic frequency and observed heterozygosity were obtained by PowerMarker (Liu and Muse, 2005).

Estimators of genetic diversity within these 16 genotypes were based on the shared alleles distance (Dps) in pairwise comparisons. The estimates of genetic distance were calculated in MicroSat as the parameter: [1- Dps]. The cladogram trees were obtained by the PHYLIP program with Neighbor-Joining method based on the genetic distance matrix obtained as described above. Trees were drawn using Tree View.

Results and Discussion

Among 556 amplifiable markers, 80 pairs of primers were suitable for 52°C 'touchdown' PCR reaction, and the others worked with 56°C 'touchdown'. The number of primers in the five classified rating groups was 281, 95, 85, 28, and 67 from excellent to not-good, respectively. Out of the 556 functional markers in the original screen, the 281 (50.5%) markers classified in the excellent group gave reliable unambiguous results and were used for the analysis of genetic diversity among the 16 genotypes. A total of 235 (42.3%) markers showed polymorphisms. The percentage of polymorphism detected in this study was relatively high when compared to other DNA markers such as RFLPs and RAPDs (He *et al.*, 2005). However, only 83 of the polymorphic markers (29.5%) were classified among the 281 excellent markers, which was lower than expected. There were 42 (44.2%) and 49 (57.6%) polymorphic markers from good and fair groups, respectively. The primers that generated ambiguous bands and no amplification within one or more genotypes produced higher estimates of polymorphism. Therefore, we used the data generated by the 461 markers from excellent, good, and fair groups with reasonable amplification quality and repeatability detected by GeneMapper. The average heterozygosity estimated from 556 SSR markers was 0.225 with a range of 0 to 0.992 for

Table 4. Genetic distances among 16 cultivated peanut genotypes calculated with data from 281 SSR markers.

	Tif-runner	GTC20	GTC9	Geor-ganic	Tif-guard	C7241925	GAGreen	GPNCWS13	NC6	NC94022	Span-cross	TNRed	Tifton8	PE1	PE2	SunOleic
Tifrunner	0.000															
GTC20	0.150	0.000														
GTC9	0.168	0.078	0.000													
Georganic	0.053	0.151	0.169	0.000												
Tifguard	0.086	0.140	0.160	0.110	0.000											
C7241925	0.059	0.142	0.167	0.087	0.033	0.000										
GAGreen	0.095	0.116	0.130	0.097	0.071	0.069	0.000									
GPNCWS13	0.094	0.157	0.160	0.119	0.088	0.082	0.115	0.000								
NC6	0.089	0.149	0.157	0.099	0.103	0.090	0.105	0.063	0.000							
NC94022	0.128	0.141	0.140	0.131	0.128	0.125	0.095	0.110	0.092	0.000						
Spancross	0.131	0.106	0.132	0.132	0.127	0.118	0.114	0.114	0.101	0.125	0.000					
TNRed	0.153	0.141	0.153	0.149	0.129	0.126	0.119	0.149	0.136	0.134	0.117	0.000				
Tifton 8	0.002	0.150	0.168	0.054	0.085	0.058	0.094	0.093	0.088	0.129	0.130	0.152	0.000			
PE1	0.122	0.130	0.122	0.127	0.106	0.101	0.088	0.087	0.094	0.120	0.105	0.120	0.121	0.000		
PE2	0.137	0.111	0.113	0.128	0.128	0.128	0.112	0.123	0.118	0.126	0.108	0.108	0.136	0.070	0.000	
SunOleic	0.089	0.106	0.122	0.101	0.078	0.077	0.055	0.113	0.102	0.101	0.106	0.114	0.089	0.094	0.103	0.000

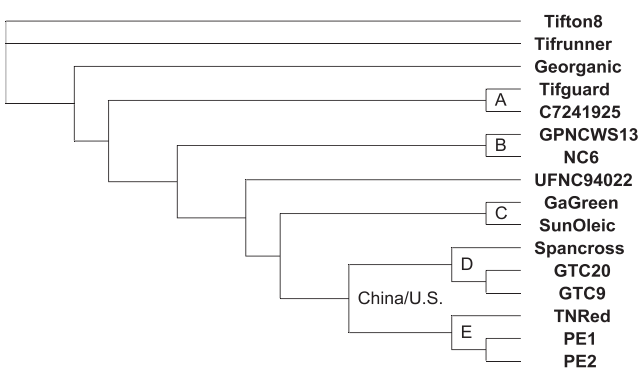

Fig. 1. The horizontal rectangular cladogram of 16 cultivated peanut genotypes based on the genetic distance calculated from 281 SSR markers data by Neighbor-Joining method. A, B, C, D, E clades or clusters and three outliers ('Tifton 8', 'Tifrunner', and 'Georganic') were formed. The assigned clusters were in agreement with either pedigree or origin.

individual SSR markers and the average of PIC was 0.209.

The average number of SSR alleles among 709 pairs of primers tested in this study was 2.5 with a range of 1 to 13 among the 16 peanut genotypes. However, only one allele was found in 410 of these SSR markers in the 16 genotypes, indicating that diversity of cultivated peanuts is very limited. Among those 125 polymorphic SSR markers from the excellent and good quality groups, 26.4% of the markers were dinucleotide GA repeat motif SSR markers, followed by dinucleotide CT (10.4%), and trinucleotide TAA (9.6%). This is in agreement with the earlier report (Ferguson *et al.*, 2004). This research validated that the dinucleotide and trinucleotide repeat motifs are the most abundant type of SSR, and dinucleotide GA repeat motif shows a higher polymorphism compared to other trinucleotide and polynucleotide repeat motifs (Moretzsohn *et al.*, 2005).

The genetic distance matrix was estimated by shared allele distance in pairwise comparisons of 16 peanut genotypes based on 281 excellent quality markers with unambiguous bands (Table 4). The genetic distances among the 16 tested genotypes varied from 0.002 to 0.169. The smallest genetic distance was between 'Tifton8' and 'Tifrunner', where both were developed in Tifton, Georgia, but no apparent similarity in genetic background (Holbrook and Culbreath, 2007; Coffelt *et al.*, 1985) and the largest genetic distance was between 'Georganic' (a typical runner-type) and 'GTC9' (a typical Spanish-type).

A horizontal rectangular cladogram of the 16 genotypes was constructed based on the genetic distance matrix with Neighbor-Joining method (Figure 1). The Neighbor-Joining method assigned the 16 genotypes into A, B, C, D, E clades or clusters and three outliers ('Tifton 8', 'Tifrunner',

and 'Georganic') (Figure 1). Basically, the assigned clusters were in agreement with either pedigree or origin. The sister lines 'Tifguard' and 'C 724-19-25' were grouped as cluster A. Two Virginia type peanuts, 'GPNCWS13' and 'NC6', were grouped as cluster B. 'Georgia Green' (GaGreen) was grouped together with the released cultivar 'SunOleic 97R' in cluster C. Cluster D included two Chinese released cultivars ('GTC20' and 'GTC9') and one U.S. released cultivar 'Spancross' (Hammons, 1970), which are Spanish-type. Cluster E consisted of two Chinese breeding lines ('PE1' and 'PE2') and another U.S. released cultivar 'Tennessee Red' (TNRed), which are Spanish-type and Valencia-type, respectively.

A total of 709 SSR markers were collected from public databases which will be a valuable asset to the peanut research community; 556 SSRs passed an initial screen and were used to characterize 16 diverse peanut genotypes. PIC scores and heterozygosity indices were calculated to assess the genetic diversity of SSR markers, and genetic distances were also estimated from shared allele distances for construction of a cladogram to illustrate the genetic relationships among the tested genotypes. Although the majority of the SSR markers used in this study have been developed and screened in other *Arachis* genotypes, polymorphism information has been expanded using different genotypes. This is obvious because different genotypes carry different SSR alleles. The previous genotypes used for polymorphism screening were mainly from South America (He *et al.*, 2003; Ferguson *et al.*, 2004; Moretzsohn *et al.*, 2004, 2005). In this study, 12 genotypes are from the U.S. and 4 genotypes are from China. The SSR markers developed by Ferguson *et al.* (2004) were not as polymorphic in this study in comparison to their report, in which 110 markers out of 192 showed polymorphism. The peanut genotypes used in their research were from different origins and different market types, so they may have had more diverse genetic backgrounds compared to the genotypes in this study.

Out of 56 designed markers developed from a microsatellite enriched library by He *et al.* (2003), 19 markers (34%) were polymorphic in their research, but 22 markers showed polymorphism in this study. Those 24 genotypes reported by He *et al.* (2005) consisted of different botanical varieties including *hypogaea, hirsuta, fastigiata, peruviana, aequatoriana,* and *vulgaris* with the origin of South America. Similar polymorphism results were also observed for the markers developed by Moretzsohn *et al.* (2004, 2005) who reported that 3 markers out of 67 designed markers were polymorphic within 5 cultivated peanut lines and 66 markers out of 271 designed markers were polymorphic within six *A. hypogaea* accessions mainly from Brazil. Although it is apparent that different sets of genotypes exhibit different levels of polymorphisms of SSR markers, the origin of DNA sequence information that was used for development of SSR markers also could be responsible for some of the observed differences. The markers developed from *Arachis pintoi* instead of *Arachis hypogaea* (Palmieri *et al.*, 2002, 2005) did not show polymorphism among the 16 genotypes in this study. Among the 97 newly developed SSR markers (Table 3), 40 markers showed polymorphisms, which is a relatively high rate of polymorphism.

This research also corroborated that the dinucleotide and trinucleotide repeat motifs were the most abundant type of SSRs, and dinucleotide GA repeat motif showed a higher polymorphism compared to other trinucleotide and polynucleotide repeat motifs (Moretzsohn *et al.*, 2005). The most frequent repeat family identified was dinucleotide GA, which was also reported by Ferguson *et al.* (2004), followed by dinucleotide CT (10.4%), and trinucleotide TAA (9.6%).

Most of the genetic relationships revealed from the cladogram by SSR alleles corresponded well to the pedigrees and origins of the genotype pairs. One of the closest genetic relationships was from 'Tifguard' and 'C 724-19-25'. These are sister lines and have been reported as near-isogenic (Holbrook *et al.*, 2008b). Although they both were developed by crossing of 'C-99R' and 'COAN', they differ greatly in reaction to the peanut root-knot nematode. 'Tifguard' is a released cultivar with a high level of resistance to peanut root-knot nematode, whereas, 'C724-19-25' is susceptible to the nematode (Holbrook *et al.*, 2008b). The largest genetic distance was between 'Georganic' and 'GTC9'. 'GTC9' belongs to the Spanish type and was developed in China, while 'Georganic' is a runner type peanut developed in Georgia. The genetic backgrounds of 'Georganic' and 'GTC9' are distinct. Two Chinese breeding lines 'PE1' and 'PE2' were grouped together in cluster E with U.S. released cultivar 'TNRed', which was unexpected. The two Chinese breeding lines belong to Virginia type, whereas TNRed is a Valencia type. The similarity of 'NC 6' and 'GPNPWIS-13', placed together in cluster B, was also not surprising since 'NC 6' was one of the parents used to develop 'GPNC WS 13' (Stalker *et al.*, 2002). However, there is no apparent close relationship between 'Tifton8' and 'Tifrunner', which had the smallest genetic distance. 'Tifunner' was developed from a cross of a component line of the cultivar 'Florunner' and PI

203396 (Holbrook and Culbreath, 2007), whereas 'Tifton-8' was developed as a Virginia-type peanut from a selection from a Spanish-type, PI 261976 (Coffelt *et al.*, 1985).

The information gained in this study has been used in genotype selection for genetic mapping population development and the markers screened in this study are also used in genotyping of the developed RILs (recombinant inbred lines). Two RIL populations have been developed from crosses of Tifrunner × GTC20 and SunOleic 97R × NC94022 (Guo *et al.*, unpublished data). Furthermore, this study will contribute to the construction of genetic linkage maps for cultivated peanut, which will be an important research goal to facilitate quantitative trait locus (QTL) analysis and gene tagging for use in marker-assisted breeding.

Acknowledgments

We thank Billy Wilson and Jake Fountain for technical assistance in the field and the members of Dr. Knapp's Laboratory particularly Drs. Tang and Ma. This research was partially supported by USDA Specific Cooperative Agreement 58-6602-6-121 with the University of Georgia, and the graduate student assistantship was partially supported by funds provided by Georgia Peanut Commodity Commission and the National Peanut Board. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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