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Development of trinucleotide (GGC)n SSR markers in peanut (*Arachis hypogaea* L.)

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Abstract Cultivated peanut (*Arachis hypogaea* L.) is an oilseed crop of economic importance. It is native to South America, and it is grown extensively in the semi-arid tropics of Asia, Africa, and Latin America. Given an extremely narrow genetic base, efforts are being made to develop simple sequence repeat (SSR) markers to provide useful genetic and genomic tools for the peanut research community. A SSR-enriched library to isolate trinucleotide (GGC)n SSRs in peanut was constructed. A total of 143 unique sequences containing (GGC)n repeats were identified. One hundred thirty eight primer pairs were successfully designed at the flanking regions of SSRs. A suitable polymerase was chosen to amplify these GC-rich sequences. Although a low level of polymorphism was observed in cultivated peanut by these new developed SSRs, a high level of transferability to wild species would be beneficial to increasing the number of SSRs in wild species.

Keywords: cultivated peanut, microsatellites, polymorphism, simple sequence repeat

INTRODUCTION

Cultivated peanut (*Arachis hypogaea* L.) is one of the most important oilseed crops due to its valuable source of vegetable oil and protein. However, improving peanut production by molecular tools has been difficult because of its narrow genetic base which originated from a single and recent polyploidization event (Young et al. 1996). Simple sequence repeat (SSR) markers are more informative because they are multi-allelic, co-dominant, and abundant in plant genome, compared to other molecular marker systems, such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP). Thus, SSR markers are favored as genetic and genomic tools for plant genetic linkage mapping, diversity study, and plant breeding programs. Recent studies have shown that SSRs in different positions of a gene can play important roles in determining protein function, genetic development, and regulation of gene expression (Lawson and Zhang, 2006).

A considerable number of SSR sequences have been identified from peanut genome by several research groups (Hopkins et al. 1999; He et al. 2003; Ferguson et al. 2004; Moretzsohn et al. 2005; Proite et al. 2007; Cuc et al. 2008). SSR markers developed from these repeat sequences offer promising genetic and genomic tools in peanut research. Using SSR markers, genetic diversity of peanut germplasm has been studied in Valencia (Krishna et al. 2004), in mini-core collection (Barkley et al. 2007), and in Chinese (Tang et al. 2007) and Japanese peanut germplasm collections (Naito et al. 2008). SSR markers have been identified and characterized for association with resistance traits

such as rust and late leaf spot resistance (Mace et al. 2006), and resistance against *Ralstonia solanacearum* (Jiang et al. 2007) and *Sclerotinia minor* (Chenault et al. 2008). Genetic linkage maps with SSR markers have been constructed for diploid AA genome (Moretzsohn et al. 2005), BB genome (Moretzsohn et al. 2009), tetraploid AABB genome derived from a cross of cultivated with amphidiploids (Foncéka et al. 2009), and tetraploid AABB genome in the cultivated peanut (Hong et al. 2008, Varshney et al. 2009; Hong et al. 2010). Although an exceedingly large number of SSRs have been identified, the polymorphic SSR markers may not be sufficient for the construction of a saturated linkage map in the cultivated peanut, provide enough meaningful markers for marker-assisted selection in peanut breeding programs, or sufficient coverage of important domains of the peanut genome for functional genomics research.

A survey of peanut SSR sequences in public data either from genomic DNA or that derived from expressed sequence tags (ESTs) has shown that AG/TC repeats are predominant, followed by AC/TG in dinucleotide repeats. The AAT/TTA repeat is abundant among trinucleotide repeats in peanut (Ferguson et al. 2004; Moretzsohn et al. 2005). The trinucleotide (AAT)n SSRs also showed high frequencies in other legume species such as soybean (Gao et al. 2003) and *Medicago* (Eujayl et al. 2004). However, CCG/GGC repeat is the most common repeats in monocot species (Cordeiro et al. 2001; Morgante et al. 2002; Varshney et al. 2002). Although Zhao and Kochert (1993) have mentioned that (GGC)n microsatellites were present in peanut, no detailed information on this type of SSR was available because only a few (GGC)n SSRs were isolated either from genomic DNA or ESTs to date. Therefore, the objectives of this study were to isolate (GGC)-SSR sequences to assess their abundance in peanut genome, and to test their informative content.

Table 1. A set of 24 genotypes used for polymorphism testing.

PI number	Market type	Polyploidy	Species	Botanical variety	Original country
493581	Valencia	4X	*A. hypogaea*	*fastigiata*	Argentina
274193	Runner	4X	*A. hypogaea*		Bolivia
371521	Runner	4X	*A. hypogaea*		Israel
200441	Runner	4X	*A. hypogaea*		Japan
196635	Spanish	4X	*A. hypogaea*		Madagascar
259851	Runner	4X	*A. hypogaea*		Malawi
337293	Runner	4X	*A. hypogaea*		Brazil
338338	Valencia	4X	*A. hypogaea*		Venezuela
461427	Valencia	4X	*A. hypogaea*		China (PRC)
240560	Runner	4X	*A. hypogaea*		South Africa
162857	Virginia	4X	*A. hypogaea*		Sudan
478850	Valencia	4X	*A. hypogaea*		Uganda
442768	Runner	4X	*A. hypogaea*		Zimbabwe
482120	Runner	4X	*A. hypogaea*		Zimbabwe
471954	Valencia	4X	*A. hypogaea*		Zimbabwe
461434	Runner	4X	*A. hypogaea*		China (PRC)
319770	Runner	4X	*A. hypogaea*		Israel
502096	Valencia	4X	*A. hypogaea*	*peruviana*	Peru
602090	Spanish	4X	*A. hypogaea*	*vulgaris*	Sri Lanka
560927	Valencia	4X	*A. hypogaea*	*fastigiata*	Bolivia
476011	wild	2X	*A. cardenasii*		Bolivia
476012	wild	2X	*A. cardenasii*		Bolivia
468369	wild	4X	*A. glabrata*	*glabrata*	Paraguay
338305	wild	4X	*A. glabrata*	*hagenbeckii*	Argentina

MATERIALS AND METHODS

Construction of SSR-enriched library

A method of obtaining SSR-enriched library modified from the procedures of Kijas et al. (1994), Hakki and Akkaya (2000), and Reddy et al. (2001) was used to develop SSR markers (He et al. 2003). Briefly, genomic DNA was isolated from peanut leaves using the MasterPure^{TM} Plant Leaf DNA purification kit (Epicentre, Madison, WI). The DNA was digested by *Hind*III and *Mse*I, and the restriction fragments were ligated by corresponding adapters and amplified following the AFLP protocol (Vos et al. 1995). The biotinylated SSR probe $(GGC)_{15}$ was used to hybridize the denatured pre-amplified fragments. The hybridized mixture was added to streptavidin-coated paramagnetic beads. The DNA-probe hybrids were incubated at room temperature, and a magnetic field was applied to precipitate the beads, which were attached by SSR-containing fragments that hybridized to biotinylated probes. The SSR-enriched fragments were amplified by polymerase chain reaction (PCR), products were cloned into the TA-cloning vector pCR4-TOPO utilizing topoisomerase-mediated ligation (Invitrogen, San Diego, CA), ligated vectors were transformed into chemically competent *E. coli* TOP10 and plated onto Luria-Bertani plates (LB) with antibiotic selection. Single colonies were selected, grown overnight in LB. Plasmids were purified and sequenced.

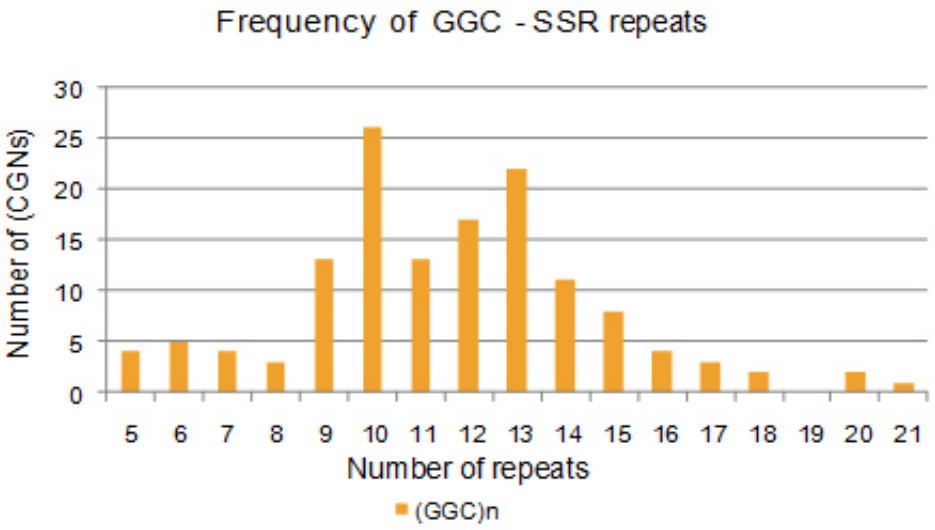

Fig. 1 Frequency of (GGC)-SSR repeats in peanut.

Marker development

SSR-containing sequences were used for primer designing using the software Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The designed primers were tested for amplification using one genotype DNA. Because amplicons are GC-rich sequences, PrimeSTAR HS DNA polymerase produced by TaKaRa Bio Inc. (Shiga, Japan) was used for PCR amplification instead of *Taq* DNA polymerase. PCR reactions were performed in 10 µl volumes, containing 1 x PrimeSTAR GC buffer (100 mM Tris-HCl, pH 8.3, 10 mM KCl, 2 mM $MgCl_2$, $(NH4)_2SO_4$), 2.5 mM of each dNTP, 0.25 U of PrimeSTAR HS DNA polymerase, 0.375 µM of each primer and 10 ng of genomic DNA. Amplifications were conducted using a DNA Engine Dyad (BioRad, CA, USA) thermal cycler, with the PCR program: 98ºC for 3 min (1 cycle), 98ºC for 10 sec, 54-60ºC for 5-10 sec, 72ºC for 1 min (30 cycles), and 72ºC for 7 min (1 cycle). The annealing temperature and time were optimized for each primer. The PCR products were separated on 6% non-denaturing polyacrylamide gels stained with ethidium bromide.

Table 2. Primer pairs designed based on the flanking sequences of SSRs.

Name	GenBank accession number	Motif	Poly-morphism	Forward primer	Tm	Reverse primer	Tm	Size
PM 601	AY526357	$(GGC)_{18}$		GAG GGA GCA CAA ATG AGG AA	64,1	TCT AGG TTC TCC CTG CAA CG	64,2	224
PM 602	AY526358	$(GA)_9$, $(GGC)_{13}$		GCT GGG ATC TTG TGG AGC TA	64,2	AAG CCG AGG AAG GAG AAG AG	63,8	208
PM 603	AY526359	$(GGC)_{13}$		GGA AGG CTG CAA AAG TAA GG	62,6	CGC GCT CAT CTT AAG TCC TA	62	164
PM 604	AY526360	$(CCG)_{13}$		ACC ACC TCT GTG CTC TTG CT	64	GCT TCT GAT GGT GGT GGT CT	64,1	241
PM 605	AY526361	$(CCG)_{10}$		AGC CCT GTC AAT CAT TGG AT	63,2	CTA GTT CGG CGG CTT CAT AG	63,6	152
PM 606	AY526362	$(GGC)_{12}$		GCG GTG GAG GCT ACT GTA AG	63,6	CGC CGT CCT TCT CTC TAT GA	64,3	240
PM 607	AY526363	$(GGC)_{13}$		GGC GAC GCA ATT GAG ACT AT	64	GCC CGT GCT ACT ATC GTC AT	63,9	173
PM 608	AY526364	$(CCG)_{17}$		AGA ACA GGC AAA ACC CAA CA	64,3	TGG TGG TGG AAT GAG TGT TT	62,9	396
PM 609	AY526365	$(CCG)_{14}$		TGC CAT CTC TCT CCC TTG AA	64,8	TGG CGA CAG TGG AGA TTA GA	63,4	196
PM 610	AY526366	$(GGC)_{10}$		TGC TCC CCT GTT GTT ACA GA	63,3	CTG TGC TCT TGC TTA GAT CTG G	62,9	159
PM 611	AY526367	$(CCG)_{14}$		ATC GCA TAA CCA CCA CCA TC	64,5	GGA GGC AAC CCC ATT TTA TT	63,5	249
PM 612	AY526368	$(GGC)_9$		CAC CTC TCG GAC GTG TCA TA	63,8	TCC GTC TTC TCC TTC TCC AA	63,8	246
PM 613	AY526369	$(GGC)_{15}$		CTC TGG CAA CGA CGA CTG TA	64,1	TCA GAA ATG GGA CCA TGA CA	64	228
PM 614	AY526370	$(CCG)_{13}$		ATA GGA TGA GCT GCC CTG TT	62,5	CCG TAA GCG TAA TGG AGC AT	63,7	216
PM 615	AY526371	$(GGC)_6$	y	AGA GAG AAT CCA TGG CGA GA	63,8	ATG CTC ACC CCC TGT CTG	63,9	214
PM 616	AY526372	$(GGC)_{10}$		TCC ATG AAC CAG CAA ACA GA	63,8	ATT CCA ACG CAA CCA ATA CC	63,7	231
PM 617	AY526373	$(CCG)_{13}$		ACG ACT CCT CCA TTG TCA CC	64	GGC GAC GCA ATT GAG ACT AT	64	227
PM 618	AY526374	$(CCG)_{14}$, $(CT)_{11}$		ATC TGC GTC AGA AGG GAG AA	63,9	GCC GAC GAG GAG ATA GTC AG	63,9	201
PM 619	AY526375	$(GGC)_8$		GGG GGT GTC TCA AAG TCT CA	64,1	CCG CGC CTA GTA TTG TCT GT	64	249
PM 620	AY526376	(GGC)16		GGA GAG GGA GCA CAA ATG AA	64,1	CAC TCC AGT GTC GTT GCA CT	64,1	184
PM 621	AY526377	$(CCG)_{11}$		CAG AGA TCA ATG GGG AAG GA	63,9	CGG TGA AAC CTG GTG GTT AG	64,2	157
PM 622	AY526378	$(GGC)_{13}$		CTC CGG GAA CTC CTC TCT CT	63,8	CTC CGC AGT CAT TCA TCT CA	64,1	224
PM 623	AY526379	$(GGC)_{17}$		GGG CTC CAT ATG CAC TTC AG	64,5	AAC CAC AGC ATC GGA GTC TT	63,6	178

PM 624	AY526380	$(CCG)_{13}$	AGG GAA AGA AAC GCA GAA CA	63,6	GAA GAT GAA GGG AGC AAC CA	64,1	209
PM 625	AY526381	$(GGC)_{10}$	GTT GGG CTT GGT GTG ATC TT	63,9	GGT CCG CAC AGG AAA AGT TA	63,8	181
PM 626	AY526382	$(CCG)_{9}$	TGT GCT CTT GCT TAG ATC TGG A	63,9	GAA GCA GGG AAA GTT TGT CG	63,6	168
PM 627	AY526383	$(GGC)_{12}$	GGG CAG CAG AGA TAG ATT GG	63,6	TGT GCT CTT GCT TAG ATC TGG A	63,9	204
PM 628	AY526384	$(GGC)_{14}$	GCA AAG AAA AAC CAC GAA ACA	63,7	TCA AAG AAC TCC CTC GGC TA	63,7	207
PM 629	AY526385	$(CCG)_{15}$	CAA CTT CAA TCC CTG CCA TT	63,7	CCA TAG AGG TAG TGA CCA GAT ACA	60,8	250
PM 630	AY526386	$(CCG)_{12}$	CTC TCC GTG GCT GCT ACT TC	64	TGG CTG GTG AGA GAC AGA GA	64	152
PM 631	AY526387	(GGC)10	GGT GAC GTG GTG AGA ACG AT	65,1	GTC CCT CAA TTT CAG CCA GA	64,1	228
PM 632	AY526388	$(CCG)_{6}$	CCA CCA AGT TGT GGT CTT GTT	63,7	AGA TCG AGG CAA GAA GCA GA	64,1	167
PM 633	AY526389	$(CCG)_{10}$, $(CT)_{10}$	TCT AAA CCC CTG ACC TCT TCC	63,2	CGT TCG GAG GAG TGA GAG AC	64	172
PM 634	AY526390	$(GGC)_{9}$	GAG GGA GCA CAA ATG AGG AA	64,1	AAC CTT TGC GGA CTG ACC TA	63,5	209
PM 635	AY526391	$(GGC)_{5}(GGT)_{2}$	CTG GTA GTG ATT CCG GCA GT	64	CTC CCC TAC CCT AGC CAA AC	63,5	211
PM 636	AY526392	(CCG)12	AAA AGA AGG GGA AAC GAG GA	63,6	CAA ATG AAT TCA ACG CGA GA	63,6	204
PM 637	AY526393	$(GGC)_{21}$	TGG AAT TGC TGC TGG TAA TG	63,5	AGG ATG AAC CAT GCA CAA AA	62,9	219
PM 638	AY526394	$(CCG)_{10}$	ACT CGT CTG CTC ATC GTC CT	64	CCT TCT CTC TCG CGT CAA GT	63,6	213
PM 639	AY526395	$(CCG)_{15}$	GGA TTA ACC GCC GTC CTT AC	64,2	GTT GGC GGA GGA TGG AAG	65,7	283
PM 640	AY526396	$(CCG)_{15}$	CGA TGA AGA CGA AGA CGA CA	64,1	CGA GGA ACT CGA ACC AGA AG	63,8	231
PM 641	AY526397	$(GGC)_{12}$	GGC GAC GCA ATT GAG ACT AT	64	ACC AGA GGG CAT TGT TGT TC	63,9	195
PM 642	AY526398	$(CCG)_{15}$	CTC TCT TTG GCT CCA TAC GC	63,7	GGC GAC GCA ATT GAG ACT AT	64	167
PM 643	AY526399	(GGC)7	GGC GAC GCA ATT GAG ACT AT	64	TCA GTT AGA GCC ACC CAT CC	64	178
PM 644	AY526400	$(CCG)_{10}$	CCG AGG GAT AAG AGT TTC CA	63,7	CGA CTC ACT ATA GGG CGA ATT G	63,9	202
PM 645	AY526401	$(CCG)_{11}$	CCT CCC CTA TGA CAC TGC TC	63,6	CTT CAA GTG AGG TGG CGA TG	65,8	155
PM 646	AY526402	$(GGC)_{13}$	GCA AAG CAA AAG TGG CTT CT	63,3	CCC TCT CTG CCC TAA ACA CA	64,1	220
PM 647	AY526403	$(GGC)_{11}$	AGC GGC TGT AAT GGA AAC AG	64	GCA ACC CAG ATT TCT TCA CC	63,4	162
PM 648	AY526404	$(GGC)_{11}$	ATA GCA TCG GTG ACG AGC TT	63,6	TCT CCC TCC AAC CAC CAC	63,8	215
PM 649	AY526405	$(GGC)_{17}$	GCG ACG CGA TTG AGA CTA TT	64,1	ACG CCT CTA TTC CGC CTA TT	63,6	158

PM 650	AY526406	(CCG)9		AAC CTC TCA ATG CCC CTT CT	63,8	GTG GTG AGG GGA AGC AGA TA	64	163
PM 651	AY526407	$(CCG)_{11}$		CAA ACG TAA CCC ATC TGT TCT TC	66,1	CGA CTC ACT ATA GGG CGA ATT G	63	184
PM 652	AY526408	(GGC)8	y	GGA AAA CCC TAA CCC CTT GA	63,7	TCT CTC TTC TCA CCC TCC TTT C	62,7	132
PM 653	AY526409	$(GGC)_{14}$		CTA TTC GAC GAG TTG CGA TG	63,3	CTC CCA ATC CCT CAG TTC AC	63,5	174
PM 654	AY526410	$(CCG)_{12}$		CGC GTT CAA GAG AGA AAA GG	63,8	GGG AGA CGA GGG AGA GTG AT	64,6	229
PM 655	AY526411	$(CCG)_{15}$		GCA TGA GAG CTC ACT GAA ACC	63,9	GGG TTG TTT CCT TTG CCA TA	63,5	202
PM 656	AY526412	$(GGC)_{11}$		GCC ACG CTC TTA AAC CTT TC	62,6	GGC GAC GCA ATT GAG ACT AT	64	238
PM 657	AY526413	$(CCG)_{11}$		AAC GAG GGG GAA GAA GAG AA	63,9	GGT GAT GCA GCA GAT TCA GA	64,1	216
PM 658	AY526414	$(GGC)_{11}$		AAT CGG AGT GCA GTG AAA CC	64	TGG CTG TGC TCT TGC TTA GA	63,8	274
PM 659	AY526415	$(CCG)_{13}$		ACG CAA AAA CCC TAA TCG TG	63,6	GGG GAA ACA GAA AGG AAA AA	61,7	219
PM 660	AY526416	$(GGC)_{12}$		CTA TTC GAC GAG TTG CGA TG	63,3	AGG TCC GCT AGG GTT TTC AT	63,5	241
PM 661	AY526417	$(CCG)_{12}$	y	GTT CTT CGC TGC GTC TCT CT	63,8	GCG TCT TCT CAA TCG TGT CTC	63,9	202
PM 662	AY526418	$(GGC)_{7}$		CGA CGC TGT TGA GAC TAT TCC	63,6	CGT GAA GGG GAT AGA TCA TTG	63,1	249
PM 663	AY526419	(GGC)16		GGG AAG AGG AAG AGG AGG AA	63,5	GCA AAG GAA GGG ACA GAC AG	63,7	172
PM 664	AY526420	$(GGC)_{18}$		AAG CTC CAT TAA CGG TGA CG	63,7	AAA ACT CCG CCA TCA ACA TC	63,7	155
PM 665	AY526421	$(GGC)_{12}$		CAT GGC AAT AAC ATT CCT CCT	62,5	TTT CTG GAG CCA CCT CTG AC	64,4	206
PM 666	AY526422	$(GGC)_{10}$		ACA AGC TCC AGC TGA AAG GA	64	GCT TCT TCT CTC CAT CAC ACG	63,9	234
PM 667	AY526423	(CCG)10		AAG CAA AAC CCT AGC CCT TC	63,2	GAG TCA TTT GCG GAG GAG AG	63,9	219
PM 668	AY526424	$(CCG)_{10}$		CCT ACC ACT TGC GTC CAA AT	63,5	CGA CTC ACT ATA GGG CGA ATT G	63,9	237
PM 669	AY526425	$(GGC)_{14}$		GCA GAC ATG AAC GAC AGT GG	64,5	ACT ACC GCC GGA AAA ACT TC	64	181
PM 670	AY526426	$(GGC)_{9}$		GTG CGA GAA CAG ACC TCC TC	64	AAT CAC CGT AGC GGT GTA GC	63,9	222
PM 671	AY526427	$(GGC)_{12}$		GAG GGA GCA CAA ATG AGG AA	64,1	CCT CAC CAA CCC TCA TTC TC	63,5	206
PM 672	AY526428	$(GGC)_{12}$		TGG CTA GGA AAG GTC TTC CA	63,6	TGC CAT TCT CAT CCT CTT CC	64,1	178
PM 673	AY526429	$(CCG)_{9}$	y	CAT CGT TTC GCA CAA AGC TA	63,8	TCT TCC ATT GAG GTT GTT GC	62,6	190
PM 674	AY526430	$(GGC)_{7}$, $(GGC)_{5}$		TGC TCC ATT GAG ACA AGC TC	63,2	ATC CTC CGC AGC TTC CAC	65,4	206
PM	AY526431	(CAT)24	y	AAT ACC CTT	63,5	TGC TTC TGC	64,3	243

675			CCC CAA TCA CC		TCG ATG TTC TG		
PM 676	AY526432	$(CCG)_{12}$	GCC GGA GGA GAA ACC AAT	64	GTG CGA GAA CAG ACC TCC TC	64	216
PM 677	AY526433	$(GGC)_{10}$	GAC AAG GAG CAT GAC GGA AG	64,8	TCT CTC CCG ACA CAA ATT CC	64	233
PM 678	AY526434	$(CCG)_{13}$	TGT GCT CTT GCT TAG ATC TGG A	63,9	GAG GGA GCA CAA ATG AGG AA	64,1	221
PM 679	AY526435	$(GGC)_{13}$	GGC AGG GAA CCT GAA ATG TA	63,7	AGA GAA GAA GAC GCG AGA CG	63,8	179
PM 680	AY526436	(GAAA)6	TGG ACT AAA AAT GGG CTT CG	63,7	TTC TCC CCG TTC TCT CAC TC	63,3	235
PM 681	AY526437	$(CCG)_{16}$	GGT GAG AGA GGG AGC ATC AC	63,9	CCT ATC TTT CCC GTC ACG TC	63,3	174
PM 682	AY526438	(GGC)5	GAC CGA AAT CGA GAT GAA CC	63,3	TCT GCT TCT GTT TCC CAT CC	64,1	235
PM 683	AY526439	$(CTT)_8$, $(CCG)_{10}$	CAC ACC GCC TTC TTC TTC TT	63,2	ATG CAG GTG TCG GAT CTT TC	64	210
PM 684	AY526440	$(CCG)_{10}$	AGT GCG AGA GAG GAG ACT CG	63,9	GTG GCG GAG GAG GGT AGT AG	64,7	174
PM 685	AY526441	$(CCG)_{10}$	ACG AGC TTC TCC CTC TCT CC	63,9	TCC GGG AAA CTC AAC AGA AG	64,1	156
PM 686	AY526442	$(CT)_8$, $(CCG)_{10}$, $(CT)_{10}$	CGC AAA CTT CTT TCC CTT CA	64	CGT TCG GAG GAG TGA GAG AC	64	222
PM 687	AY526443	$(GGC)_{10}$	TGA TAA AGG AGG CGT CTT GG	63,9	CTG TGG GGG TTC TCA CTG TC	64,7	183
PM 688	AY526444	$(CCG)_{13}$	GAG CTT GGT AAC GCC CAT AG	63,4	GGA GGA GGT CCA TTT GGA TT	63,9	187
PM 689	AY526445	$(GGC)_{13}$	TAG AAA GGC TTC CTC CAT GC	63,1	GAC ACC GCA ATC TGA CCT TT	64	172
PM 690	AY526446	$(GGC)_{10}$	AGA AAG AAT GGG CAG CAA AA	63,5	GAT TGG CTG GTG GAT CAC TT	63,9	204
PM 691	AY526447	$(CCG)_{11}$	TGC TTT TGT CCA AGG CTT CT	63,7	TGG GAC TAT TCG ACG GAG TT	63,3	216
PM 692	AY526448	(CCG)13	GAA CGC GTG ATG AAG GAA AT	63,9	GTC GTC CCT CTT TGT GTC GT	64,1	233
PM 693	AY526449	(GGC)9	AGC AAA GCA AAA GTG GCT TC	63,3	CCT TGA GTA TGG CTG GCT TC	63,6	174
PM 694	AY526450	$(GGC)_{14}$	AGT CCT GCA GGT TTA AAC GAA	62,5	TTG TTC GAC CTG TGC TCT TG	64,1	155
PM 695	AY526451	$(GGC)_{12}$	TGA GGG AAG AGA AGG AGA AGG	63,6	CGC TAT CCT GAG GAG GAC AC	63,7	236
PM 696	AY526452	$(GGC)_9$	AAA AGT TGA CCC ACC CAA CC	64,7	TGG TAT TGC TCC CCA CTC TC	64	197
PM 697	AY526453	$(CA)_7$, $(CCG)_{12}$	CTT TCC TCA GCC TCT GAA CG	64	CGA CTA GTT GCC GAA TGA TG	63,2	243
PM 698	AY526454	(GGC)6	TTT TGA GTA GGA ACC TGA GAG TTG	62,4	ACC TCT GGC GAC ACC ATC	64	151
PM 699	AY526455	$(GGC)_{14}$	GAG AGG GAG CAC AAA TGA GG	63,7	TCA CGA ACA TCC TCT CGT CTC	64,4	219
PM	AY526456	$(CCG)_{10}$	CAT CTG ACC	64,3	GGA ACA TGA	64,1	205

700			TGT CAC CAT CG		ACA GAG AGC AAC A		
PM 701	AY731521	(GGC)20	GAG GGA GCA CAA ATG AGG AA	64,1	ATC TGG ATT ACC AGC CAC CA	64,2	239
PM 702	AY731522	(CA)7, (GGA)5	CTC ACC TTC GCA AAT CAC CT	64,1	ACC CCC TCT CAC TCT CCA TT	63,8	217
PM 703	AY731523	(CCG)10	CAA GTC GCC GTT GCG TAG	65,6	CTT TTC CTT CTC GCG TTC TG	63,8	195
PM 704	AY731524	(GGC)16	AGT CGG TCG TTG AAA ACA GG	64	AGT GCT CAG CCT TTC ATT CC	63,3	225
PM 705	AY731525	(GGC)13	AGG TGA CGA ACC AAA ACG TC	63,9	TCA TTT CCA CCC CAC TAT CC	63,4	241
PM 706	AY731526	(CCG)14	CCA CCC TCG TCG ATC TGT	63,9	GGA GAG GGA GCA CAA ATG AG	63,7	207
PM 707	AY731527	(GGC)10, (AAG)11, (GGC)10	CTA TTC GAC GAG TTG CGA TG	63,3	GTG GTG TTG TCG CCG ATA G	64,2	217
PM 708	AY731528	(GGC)6	GAG GAA GTC TCG GAG GAG GT	63,7	CTA TTC GCC TCA ATC CCT CA	63,9	224
PM 710	AY731529	(CCG)9	GCA AAG CCT TGG ATC CTT TT	64,5	CTT GGC GTT TCT CTC TCT CC	64,3	219
PM 711	AY731530	(CCG)9	GCA AAG CCT TGG ATC CTT TT	64,1	CTT GGC GTT TCT CTC TCT CC	63	155
PM 713	AY731531	(CCG)6(AC G)2(CCG)5	GGA GAG AGA AGG GGT TGC AG	65,1	GAG AGG GAG CAC AAA TGA GG	63,7	250
PM 714	AY731532	(CCG)13	TTC ATT TCC CCT CTC CTC CT	63,7	CCC CTT CTC CTG GCT TAG AT	63,3	162
PM 715	AY731533	(CCG)12	CAT TGA GGC AGA GAG CTT CC	64	ATG GCA AAC GCT TTT CTC TG	64,1	212
PM 716	AY731534	(CCG)7	TCT CTC CGT CCA AGA GCT TC	63,6	ACG ATG GAA CCC TCT GTC AC	64	179
PM 717	AY731535	(CCG)15	AGG ATC AAG AAA AGG CAG CA	63,7	GGC GAC GCA ATT GAG ACT AT	64	164
PM 718	AY731536	(CCG)13	GAA TGT CCA GCG AGA AGG AG	63,9	TGG GGA GAG AAA ACT TGC AG	64,2	161
PM 719	AY731537	(GGC)11	CTT CCA CGT TCA CAC AAT GG	64,1	GGA GAG AGA GGA CGA AGA CG	63,1	222
PM 721	AY731538	(GGC)10	GAG GGA GCA CAA ATG AGG AA	64,1	TAC GGT GCA TAT GCC AAA GA	63,9	243
PM 722	AY731539	(GGC)13	TGG TCA AAA GGG GTA TCT GG	63,6	AAG GTC CGC TAG GAT TTT CA	62,4	168
PM 723	AY731540	(GGC)11	TGG CTA GGA AAG GTC TTC CA	63,6	ATC CTC TTC CTC TGC TGC AA	64	168
PM 724	AY731541	(CCG)2CC(GGC)5C G(CCG)4	ACG AGC TTC TCC CTC TTT CC	63,7	ACG CCA ATT TGG GAA CTT TT	64,2	161
PM 725	AY731542	(CCG)13	AAT GAG CCC GCC ACT TTA G	64	CGT CAC TCA TCA CCA TCT CG	64,5	199
PM 726	AY731543	(CCG)20	CCT TAA GCG GAA AAC CAT TG	63,1	CTC GCG GAT CTC TCT TTC TG	64,1	233
PM 727	AY731544	(CCG)8	CGA GGA TCT CGA AGG GAT GT	65,4	CAA TAA CCA GCA AGC AGC AA	63,8	177

PM 728	AY731545	(CCG)9		TTC CTG CAT TTC TCT CTT CCT C	63,5	AGC TCC GCA ACG AAA TAG G	64,1	173
PM 730	AY731546	(CCG)10		CCA TCG TCG TCC TCA TCA T	63,7	TAG AAT CAT GCC CGC ATA CA	63,9	151
PM 731	AY731547	(CCG)13		AGT CAC CGC ATC GCA CTC	65,4	CTG GGC TGG AAC TCA AAC AC	64,6	240
PM 733	AY731548	(CCG)11		CCA AAT CTT CTC ATC AAG TTG C	62,4	GAG AGG GAG CAC AAA TGA GG	63,7	206
PM 734	AY731549	(GGC)12		CTC GAC GCT GCT CTG TCT C	64,3	CGT TTC ATC CGA ATT CAC CT	63,7	228
PM 735	AY731550	(GGC)10	y	TTA CTG GGT TAG GCG GTG AC	63,7	TCC TCT AAA GGC ACC AAA CG	63,9	154
PM 736	AY731551	(GGC)14		GAG AGG GAG CAC AAA TGA GG	63,7	ACA CCG AAC AGC TTC ACT CC	64,3	212
PM 737	AY731552	(GGC)10, (GGC)10		GAG CCC CCT TCT CTC TCT TC	63,3	CGT CAT CCT CAT CGT GAA TC	63,1	275
PM 738	AY731553	(CCG)13		GCA CAG CAC CCT CTC TAT TTG	63,6	TGA GGG AAG AGA AGG AGA AGG	63,6	188
PM 739	AY731554	(GGC)11		GAA TCA TTT GCG GAG GAG AG	63,6	CAC TAG CGG AAG TCG CTG TC	64	168
PM 740	AY731555	(GGC)13		TAG AAA GGC TTC CTC CAT GC	63,1	GAC ACC GCA ATC TGA CCT TT	64	172
PM 741	AY731556	(CCG)15		AGG ATC AAG AAA AGG CAG CA	63,7	GGC GAC GCA ATT GAG ACT AT	64	164
PM 742	AY731557	(CCG)14		CTC AGA TCA AAG CAG CAC CA	64,2	TTC GTC CAG GGG TGG TAT TA	63,9	177
PM 743	AY731558	(CCG)6		CCC AGA TCC GAG AGA ATG AG	63,7	TAA CAG GGA CGG TTT GGT TG	64,5	161

Control-1 Normal CN explant cultured on MS + 1 mg l^{-1} of BA.
Control-2 Preconditioned CN explant cultured on MS + 1 mg l^{-1} of TDZ.
Each experiment consisted of three replicates and data were represented as Mean ± S.E with significance being determined at $P < 0.05$ level using DMRT.

Plant material and DNA extraction

A subset of 20 genotypes was selected from the mini core collection in peanut (Holbrook and Dong, 2005). Four wild genotypes were added to this material panel for the polymorphism test. The panel represents diverse genotypes collected from both different original countries and botanical varieties (Table 1). Total genomic DNA was isolated from young leaves of each plant using the same DNA purification kit as described above. The DNA concentration was diluted to 10 ng/µl for PCR amplification.

RESULTS

A total of 768 clones from the (GGC)n SSR-enriched library were sequenced, of which 156 (20.3%) contained SSRs. After removing redundancy, 143 unique SSR sequences were deposited in GenBank (accession number AY526357 - AY526456; AY731521 - AY731558). Using $(GGC)_{15}$ as a probe, the isolated SSRs were all GGC/CCG repeats except one containing CAT/GTA repeats. Among (GGC)n SSRs, 90.6% were perfect repeats, 3.6% imperfect, and 5.8% were compound repeats. The number of (GGC) repeats was in the range of $(GGC)_5$ to $(GGC)_{21}$, with 80% repeat number between $(GGC)_9$ and $(GGC)_{15}$. The frequency of (GGC)n repeats showed a bell-shape distribution (Figure 1).

One hundred forty three primer pairs were designed based on the flanking sequences of SSRs. The primer sequences, their melting temperature (Tm), and product size are listed in Table 2. Using regular *Taq* DNA polymerase, only 47.1% of the primer pairs yielded amplicons. This is due to the fact that GC-rich sequences can form a complex secondary structure preventing PCR amplification. In order to resolve the complex structure formation, some additives and enhancing agents including dimethyl sulfoxide (DMSO), betaine, formamide, and glycerol were used in many reports (Henke et al. 1997; Kang et al. 2005; Musso et al. 2006). In this study, the PrimeSTAR DNA polymerase (TaKaRa Bio Inc. Japan) was used because it is especially useful in the PCR amplification of GC-rich sequences. Therefore, of the 143 primer pairs, 138 could produce amplicons with this polymerase and its specific buffer, and only 5 (PM630, PM694, PM695, PM711 and PM714) did not show any PCR products for both wild and cultivated genotypes.

Further, of the 138 (GGC)n SSRs, the majority of them detected a polymorphism among 4 wild genotypes (Figure 2a), while only 6 revealed variation among 20 cultivated accessions (Figure 2b). The level of polymorphism detected by (GGC)n markers was surprisingly low in this study.

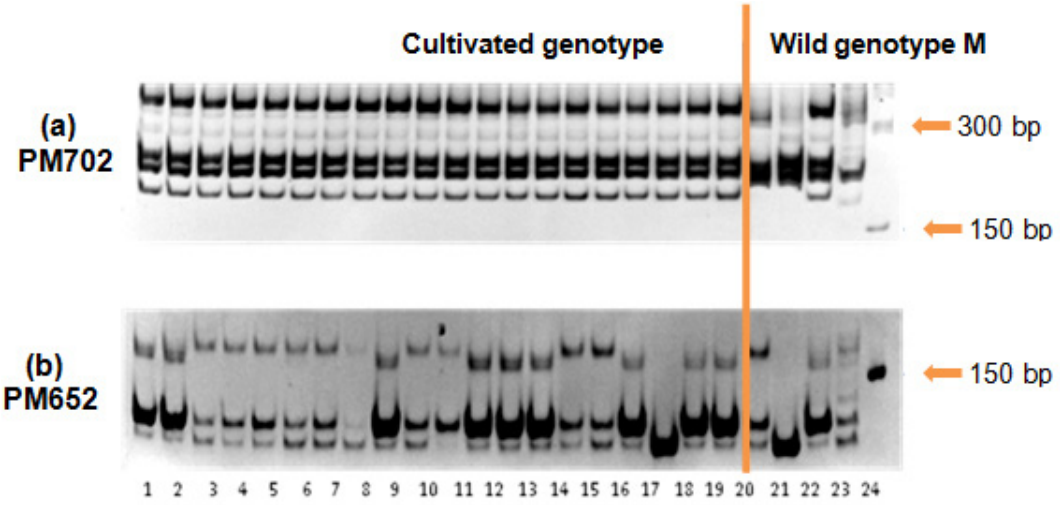

Fig. 2 Polymorphic (GGC)n SSR primers in 24 selected peanut genotypes. (a) (GGC)n primer PM702 detected a polymorphism among 4 wild genotypes, but not in cultivated genotypes. (b) Polymorphism was detected by primer PM652 in both cultivated and wild species.

DISCUSSION

The enrichment approach was used to isolate trinucleotide SSRs using $(GGC)_{15}$ as a probe, with the efficiency of isolation being as low as 20.3%. Our previous report on isolation of dinucleotide GA/CT repeat SSRs (He et al. 2003) showed a high efficiency (61%) of capturing sequences containing SSRs using the similar approaches. Low efficiency in this study may indicate that there is less abundance of (GGC)n SSRs in peanut genome, thereby lessening the chance for the probe to be hybridized with DNA sequences containing GGC/CCG repeats.

The low frequency of (GGC)n SSR in this study, as well as in other legume species, could be explained by two facts (Mun et al. 2006). The methylation of cytosine could have increased the rates of mutation to thymine, or the (GC)n repeats were selected against due to the increased stability of (GC)n hairpin structures. However, (GGC)n SSRs were predominant in monocot species. It is believed that the majority of variation of repeats in a SSR locus has resulted from slippage during DNA replication (Levinson and Gutman, 1987). But, strand-slippage theories alone are insufficient to explain the differential abundance of specific repeat types in different genomes (Mun et al. 2006). In rice, (GGC)n SSRs were observed preferentially in exons (Cho et al. 2000), while Mun et al. (2006) suggested that a positive selection pressure, such as a preference of codon usage in exons or a regulatory effect of

specific repeats in noncoding regions, may underlie the taxa-specific accumulation of certain repeat types. The taxa-specific SSRs originated after divergence of legume from monocot (Mun et al. 2006).

In three previous reports on peanut, the frequencies of repeats showed a bias towards low number in both dinucleotide and trinucleotide SSRs (Moretzsohn et al. 2005; Proite et al. 2007; Guo et al. 2009). These results were calculated from the combination of all types of SSR repeats. The mutation rate of SSRs increases with repeat number, but long SSRs in eukaryotic genomes have a mutation bias to become shorter SSRs (Hong et al. 2007). The SSRs with repeat number less than a certain threshold are stable during mitosis and meiosis, whereas above a certain threshold the SSRs become extremely unstable as shown in humans (Strachan and Read, 1999). The current result was derived from one type of SSR repeat (GGC)n. The bell-shape (no bias to either end) distribution of repeat number in (GGC)n SSRs in this study might suggest a very low and random mutation rate of all repeats in (GGC)n SSR in the peanut genome.

Optimum annealing times could also improve PCR amplification of GC-rich sequences. Mamedov et al. (2008) pointed out that shorter annealing times are not only sufficient but also necessary for efficient PCR amplification when GC-rich templates are being used. They demonstrated that 3-6 sec was sufficient, but depended on annealing temperature. When annealing time was increased, a smear was observed when products were separated using gel-electrophoresis. The manufacturer of PrimeSTAR DNA polymerase also recommended using 5 sec as annealing time. The annealing time was optimized in this study for those GGC-SSR primers. We have tested various annealing time from 5 sec to 10 sec and annealing temperature from 54 to 60ºC. The result showed that annealing time (10 sec) and annealing temperature (58ºC) were suitable for amplification of most primers. When annealing temperature was increased, more bands appeared compared to low temperatures.

A comparison of trinucleotide with dinucleotide repeats for polymorphism in peanut has resulted in different opinions by several peanut research groups. Mace et al. (2006) have reported that markers for dinucleotide repeats tend to detect a greater number of different alleles than trinucleotide repeat markers. Proite et al. (2007) also stated that dinucleotide repeats are more polymorphic than trinucleotide repeats. However, Cuc et al. (2008) reported otherwise, stating that trinucleotide SSRs showed higher allele numbers than dinucleotide SSRs. In this study, only 4.3% GGC SSRs were polymorphic based on 24 diverse genotypes. Compared to our previous results showing 33.9% polymorphic markers in dinucleotide SSRs (He et al. 2003), the current result corroborates that dinucleotide SSRs detect more DNA variation than trinucleotide SSRs.

To date, a large number of SSRs have been detected in peanut, but only about 10% of them are polymorphic (unpublished data). The effort has been made to obtain SSR markers from both genomic and EST sources. The (GGC)n SSRs identified in this study represents a new type of SSR markers in peanut with related insights into their abundance and information utility. Despite the low number of polymorphic markers in cultivated peanut, two SSR markers were mapped to an existing linkage map of cultivated peanut (data not shown). A high level of transferability of (GGC)n SSRs to wild species facilitates the construction of genetic linkage maps in wild species, and accelerates the introgression of wild useful genes into cultivated peanut.

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