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Genetic Diversity Assessment and Phylogenetic Analysis of Peanut (*Arachis hypogaea* L.) in RDA Genebank Collection using SSRs

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Abstract - It is very crucial to evaluate the genetic diversity of peanut genetic resources for identification of peanut germplasm accessions and variety improvement. Cultivated peanut generally has two subspecies, *hypogaea* and *fastigiata*. In this study, we identified peanut into three plant types, virginia (var. *hypogaea*), spanish (var. *vulgaris*), and valencia (var. *fastigiata*). Former one belongs to ssp. *hypogaea* and latter two are involved in ssp. *fastigiata*. Twenty SSR markers were used to assess the genetic variation of three sets, *hypogaea*, *vulgaris*, and *fastigiata*, respectively. Out of variety-specific SSR primers tried in this study, ten pairs of SSR primers showed polymorphisms. Each accession could be identified by a specific set of polymorphic SSR primers, and allele number was evaluated among accessions, with an average of 6.7 in var. *hypogaea* and 5.4 in var. *vulgaris* and *fastigiata*. For evaluation of genetic diversity, gene diversity ranged from 0.336 to 0.844 and PIC (polymorphism information contents) ranged from 0.324 to 0.827 were investigated. Dendrograms based on genetic distances were constructed, which showed the existence of three different clusters. And these three different clusters might be associated with the genes involved in three plant types. The results also suggested that there were plentiful SSR polymorphisms among peanut germplasm accessions in RDA (Rural Development Administration, Korea) Genebank and SSRs might play an important role in evaluating peanut accessions and cultivar improvement.

Key words - Peanut, SSR, Variety, *Hypogaea*, *Vulgaris*, *Fastigiata*

Introduction

Peanut or groundnut (*Arachis hypogaea* L.) is an important crop for oil and protein source. It is native to South America but is now cultivated in over 80 countries between 40°N and 40°S in tropical and warm temperate regions of the world. Cultivated peanut has four botanical types, *hypogaea*, *hirsuta*, *vulgaris*, and *fastigiata* (Krapovickas and Gregory, 1994). The first two botanical types belong to subspecies *hypogaea*, and the latter two to subspecies *fastigiata* based on their morphological traits and growth habits (Krapovickas and Gregory, 1994). Botanical types have their specific morphological characteristics, such as all botanical types in ssp. *hypogaea* being without floral axes on main stem and prostrate habit while those in ssp. *fastigiata* having main stem fertility and erect plant habit. In this study, we couldn't discriminate between *hypogaea* and *hirsuta*, so we identified all the accessions into three types. But there are many intermediate types (Stalker and Simpson, 1995) and they don't fit readily into either of the two recognized subspecies (Williams, 1989). Evaluation of plant phenotypes requires lots of field, labour and materials and takes long time. So, we think using molecular marker coupled with morphological characteristics to determine plant type is very useful (Scheef *et al*., 2003) to choose a variety for peanut breeder or farmer. Moreover botanical type-specific markers will be useful in genotyping, germplasm management and evolutionary research. Although there're over 3,100 varieties of peanut germplasms in RDA genebank, only 320 varieties have been discriminated by plant type (Yi *et al*., 2009).

Despite of the economical importance, peanut molecular genetics and genomics have remained at the beginning stage because of the availability of limited molecular markers. Although the morphologic traits and physiologic characteristics show significant variation in the three botanical types, both biochemical and molecular marker analyses have appeared in

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very low level or even no genetic diversity among the peanut varieties (Hilu and Stalker, 1995; Kochert *et al*., 1991; Kochert *et al*., 1996; Singh *et al*., 1993). So, it is necessary to develop more molecular markers in peanut for germplasm management and cultivar improvement.

In this study, ten SSR markers were used to study the level of genetic variation diversity for the three botanical types of *A. hypogaea*. One hundred twenty six accessions of peanut germplasms were regenerated and evaluated. We also studied the potential of these markers to discriminate the accessions of peanut germplasms by confirming the association of markers with phenotype related to plant type.

Materials and Methods

Plant materials and genomic DNA extraction

A list of peanut accessions used in this study is given in Table 1. Genomic DNA was extracted from green leaves of peanut grown for 30-40 days in the field. DNA was extracted using the modified CTAB method in each accession (Dellaporta *et al*., 1983). The extracted DNA concentration and relative purity was adjusted to 20 ng/uL and checked using Nanodrop ND-1000 (Dupont Agricultural Genomics Laboratory).

SSR amplification

The "M13 tail at its 5' end" PCR method was used to measure the size of the PCR products (Schuelke, 2000). PCR amplification was carried out in a total volume of 20 uL containing 2 uL of genomic DNA (10 ng/uL), 0.2 uL of the specific primer (10 pmol/uL), 0.4 uL of M13 universal primer (10 pmol/uL), 0.6 uL of normal reverse primer, 2.0 uL of 10XPCR buffer (Takara, Tokyo, Japan), 1.6 uL of dNTP (2.5 mM), and 0.2 uL of Taq polymerase (5 unit/uL; Takara). Conditions of the PCR amplification were as follows; 94℃ (3 min), followed by 30-33 cycles at 94℃ (30 s), 50-55℃ (45 s), 72℃ (45 s), then 15 cycles at 94℃ (30 s), 53℃ (45 s), and 72℃ (45 s), and final extension at 72℃ for 20 min. PCR was carried out in PTC-220 thermocycler (MJ Research, Waltham, MA, USA). The PCR products of three microsatellites were mixed together in a ratio of 6-FAM:HEX:NED (fluorescent dyes)=1:3:4, which was varied depending on the amplification intensity for individual markers as determined on an ABIPRISM 3130X1 Genetic Analyzer (Applied Biosystems). PCR products labeled with HEX and NED were added in higher amounts, and those labeled with FAM were added lower amounts because of the different signal intensities of these fluorescent dyes. The mixed PCR product of 1.5 uL was combined with 9.2 uL of Hi-Di formamide and 0.3 uL of an internal size standard, Genescan-500 ROX (6-carbon-rhodamine) molecular size standards (35-500 bp). The samples were denatured at 94℃ for 3 min and analyzed with an ABI PRISM 3130 X 1 Genetic Analyzer (Applied Biosystems). Molecular weights, in base pairs, for microsatellite products were estimated with Genescan software ver. 3.7 (Applied Biosystems) using the local Southern method. The individual fragments were assigned as alleles of the appropriate microsatellite loci with Genotype software ver. 3.7 (Applied Biosystems).

Data analysis

PowerMarker version 3.25 (Liu and Muse 2005) were used to measure the variability at each locus; the observed heterozygosity (H_O), the gene diversity/expected heterozygosity (H_E), and the polymorphism information contents (PIC). The number of alleles was calculated, and the PIC (polymorphism information content) as estimated from the equation $PIC=1-\sum_i P_i^2$, where P_i is the frequency of the ith allele in the sample examined (Anderson *et al*., 1993). A phylogenetic dendrogram was constructed using the unweighted pair-group method with arithmetic averaging (UPGMA) in the NTSYS-pc program (Rolf, 2000) and bootstrapping with Winboot (Yap and Nelson, 1996). Each amplified primer was treated as a unit character and scored in a binary code of either 1 or 0 for presence or absence, respectively. Genetic variation was calculated within species and genetic similarity between species with Microsoft Excel using the NTSYS dataset.

Results and Discussion

Variations of genetic diversity in accessions of peanut germplasm in RDA genebank

Out of twenty SSR primers, ten could detect polymorphisms in the 126 accessions of peanut germplasms maintained at RDA genebank (Table 1). The PCR amplification produced repeatable amplicons in the expected molecular size range.

Table 1. The accessions used for detection of DNA and morphological polymorphisms.

Botanical Type	Country Origin	Plant Introduction Number	Botanical Type	Country Origin	Plant Introduction Number	Botanical Type	Country Origin	Plant Introduction Number
Hypogaea	India	172511	*Vulgaris*	Israel	184986	*Fastigiata*	Uganda	172489
(Virginia type)	Unknown	110342	(Spanish type)	Unknown	110345	(Valencia type)	Brazil	172493
	Unknown	110329		Japan	184991		India	172722
	Honduras	172501		Unknown	030740		India	172720
	Pakistan	172589		Korea	171378		Brazil	110358
	Brazil	172440		Japan	184993		Paraguay	191334
	Korea	172414		USA	172648		Zimbabwe	172513
	Korea	110355		India	172785		Korea	172412
	Korea	030859		India	172783		India	172689
	Korea	172549		Korea	172396		China	184973
	Malaysia	184994		Unknown	172646		Brazil	191251
	India	172567		Unknown	110336		Peru	185093
	India	172746		India	172788		Israel	172638
	Zambia	184976		Argentina	184985		Philippines	172587
	USA	172593		Korea	144016		Brazil	191282
	Japan	110356		Unknown	030540		Madagacar	191319
	USA	172459		Unknown	030586		Brazil	185038
	Korea	172812		Thailand	185096		Bolivia	185043
	Sudan	172457		Brazil	191316		Bolivia	172530
	Unknown	030589		Unknown	172674		Pakistan	185002
	Korea	181768		USA	172644		Zambia	184975
	Unknown	181802		Korea	172406		Zimbabwe	185053
	Peru	185094		Madagascar	191304		Mexico	185084
	Vietnam	185040		Unknown	187840		Bolivia	191338
	Unknown	181870		China	185047		Peru	185092
	India	172574		China	172830		Jamaica	184983
	Unknown	187837		Zambia	185051		Zimbabwe	185118
	India	172697		Unknown	030703		Argentina	185005
	Unknown	187835		Unknown	030701		Brazil	185023
	USA	172592		Mexico	191328		Zimbabwe	191329
	Unknown	187843		Korea	030887		Brazil	191259
	USA	172465		India	101137		Zimbabwe	185069
	Unknown	172594		USA	101164		Brazil	191530
	Brazil	185037		KOR	030933		Argentina	191504
	Argentina	191555		USA	101160		Zimbabwe	191279
	Brazil	191473		Korea	030886		Zimbabwe	185125
	Unknown	030619		Unknown	030778		Ecuador	110309
	Unknown	030987		Philippines	191321		Bolivia	191336
	Malawi	101168					Argentina	185078
	Senegal	101152					Argentina	185017
	Korea	030880					Brazil	191317
	Korea	030882					S. Africa	191306
	Korea	110243					Sudan	191348
	USA	101163						
	India	172678						

A total of 78 alleles were detected with the 10 sets of SSRs, resulting in an average of 7.8 alleles per locus (Table 3). The PM210 primer pair, which had highest number of repeat motifs and PM3 primer pairs produced the highest number of alleles (12 alleles), whereas PM35, PM42, PM45, and PM238 produced only 5 alleles. The major allele frequency (MAF)

Table 2. Peanut microsatellite markers screened for botanical type-specific markers among 126 genotypes.

Name	Sequence (5'-3') F	Sequence (3'-5') R	Repeat motif	Size
PM_3	GAA AGA AAT TAT ACA CTC CAA TTA TGC	CGG CAT GAC AGC TCT ATG TT	$(GA)_{14}$	168
PM_{35}	TGT GAA ACC AAA TCA CTT TCA TTC	TGG TGA AAA GAA AGG GGA AA	$(GA)_{18}(GAA)_2$	135
PM_{36}	ACT CGC CAT AGC CAA CAA AC	CATTCCCACAACTCCCACAT	$(GA)_{18}$	200
PM_{42}	ACG GGC CAA GTG AAG TGA T	TCT TGC TTC TTT GGT GAT TAG C	$(GA)_4AA(GA)_{14}$	202
PM_{45}	TGA GTT GTG ACG GCT TGT GT	GAT GCA TGT TTA GCA CAC TTG A	$(GA)_{16}$	101
PM_{50}	CAA TTC ATG ATA GTA TTT TAT TGG ACA	CTT TCT CCT CCC CAA TTT GA	$(TAA)_4$, $(GA)_{19}$	103
PM_{137}	AAC CAA TTC AAC AAA CCC AGT	GAA GAT GGA TGA AAA CGG ATG	$(GA)_{20}$	150
PM_{204}	TGG GCC TAA ACC CAA CCT AT	CCA CAA ACA GTG CAG CAA TC	$(GA)_{20}$	216
PM_{210}	CCG CAG ATC TTC TCC TGT GT	CCT CCT CAT CCT CTA AAC TCT GC	$(CT)_{25}$	204
PM_{238}	CTC TCC TCT GCT CTG CAC TG	ACA AGA ACA TGG GGA TGA AGA	$(CT)_{11}$	150

Table 3. Allele number, gene diversity, heterozygosity, and polymorphism detected by SSR markers in 126 *Arachis hypogaea*.

Marker	Major Allele Frequency	Allele No.	H_E (Expectedheterozygosity)	H_O (Observedheterozygosity)	PIC
PM_3	0.224	12	0.850	0.992	0.832
PM_{35}	0.319	7	0.749	0.032	0.708
PM_{36}	0.626	5	0.505	0.000	0.425
PM_{42}	0.450	5	0.644	0.725	0.576
PM_{45}	0.496	5	0.642	1.000	0.581
PM_{50}	0.307	11	0.794	0.030	0.769
PM_{137}	0.312	6	0.752	0.082	0.708
PM_{204}	0.325	10	0.734	0.000	0.688
PM_{210}	0.419	12	0.762	0.016	0.737
PM_{238}	0.399	5	0.698	0.991	0.645
Mean	0.388	7.8	0,713	0.387	0.667

ranged from 0.224 to 0.626 with a mean value of 0.388. and PIC (Polymorphism Information Contents) revealed from 0.425 to 0.832 (Table 3). The observed heterozygosity (H_O) ranged from 0.016 to 0.992 (mean=0.387), and expected heterozygosity (H_E) ranged from 0.505 to 0.850 (mean=0.713). Substantial diversity exists among accessions of peanut germplasm in morphological, physiological, and agronomic traits. This is the base for modern cultivar improvement by hybridization in future. This study checked a wide both intra- and inter-variety diversity in cultivated peanut at the molecular level among the tested accessions from three botanical types. Dendrogrames based on genetic distances were made for the three botanical types, which revealed the existence of different clusters. The phylogenetic relationships revealed was consistent with the data of plant type obtained using evaluation method by sight (Fig.1).

The total number of alleles in the 45 var. *hypogaea* accessions were sixty seven, MAF ranged from 0.278 to 0.641 with a mean value of 0.386, and PIC revealed from 0.440 to 0.827 (mean=0.681) (Table 4). The observed heterozygosity (H_O) ranged from 0.023 to 0.725 (mean=0.392), and expected heterozygosity (H_E) ranged from 0.508 to 0.844 (mean=0.722). Fifty four alleles were detected in 38 var. *vulgaris* accessions, MAF was from 0.263 to 0.636 with a mean value of 0.459, and PIC was observed from 0.419 to 0.765 (mean=0.600) (Table 5). The observed heterozygosity (H_O) ranged from 0.026 to 0.971 (mean=0.378), and expected heterozygosity (H_E) ranged from 0.500 to 0.795 (mean=0.654). On the whole, 54 alleles were counted in 43 var. *fastigiata* accessions, MAF ranged from 0.279 to 0.810 with a mean value of 0.519, and PIC revealed from 0.324 to 0.750 (mean=0.547) (Table 6). The observed heterozygosity (H_O) ranged from 0.048 to 0.977 (mean=0.389), and expected heterozygosity (H_E) ranged from 0.336 to 0.784 (mean=0.600).

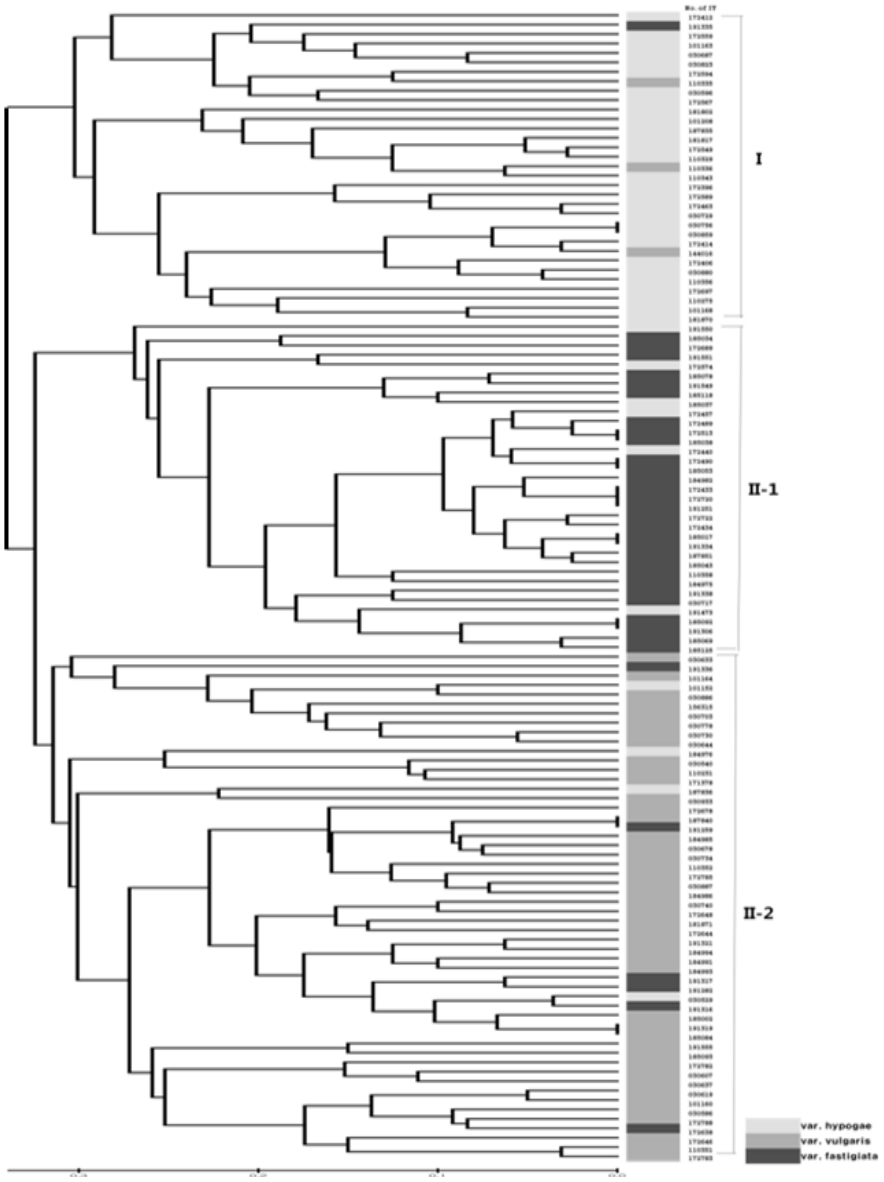

Fig. 1. Dendrogram of cluster analysis on SSR amplification profiles and plant types evaluated by visual check for the three sets of accessions of *A. hypogaea* based in RDA genebank.

The highest allele number was observed among accessions in var. *hypogaea* (6.7), followed by var. *vulgaris* and *fastigiata* (5.4). PIC (Polymorphism information contents) value was maximized in var. *hypogaea* (0.681), followed by var. *vulgaris* (0.6000) and var. *fastigiata* (0.547). This results indicated the genetic diversity in cultivated peanut was much richer at the molecular level when compared with that in the morphological levels. The genetic diversity at molecular level of Korean rice breeding parents observed by Song *et al*. (2003) was also ampler than that at morphological level.

Relationships of peanut germplasms obtained from SSR profiles

In this study, we identified peanut into three plant types, virginia (var. *hypogaea*), spanish (var. *vulgaris*), and valencia (var. *fastigiata*), because we couldn't discriminate between *hypogaea* and *hirsuta*. A phylogenetic dendrogram based on the SSR profiles showed two major clusters with genetic similarity 0.34 ; one is estimated for var. *hypogaea*, another is for var. *vulgaris* and *fastigiata* (Fig.1). The former clade, *Arachis hypogaea* var. *hypogaea* involving 33 accessions

Table 4. Allele number, gene diversity, heterozygosity, and polymorphism detected by SSR markers in 45 *Arachis hypogaea* var. *hypogaea* accessions.

Marker	Major Allele Frequency	Allele No.	H_E (Expectedheterozygosity)	H_O (Observedheterozygosity)	PIC
PM_3	0.284	10	0.812	1.000	0.789
PM_{35}	0.333	7	0.749	0.000	0.707
PM_{36}	0.641	5	0.508	0.000	0.440
PM_{42}	0.375	5	0.681	0.725	0.619
PM_{45}	0.500	4	0.658	1.000	0.607
PM_{50}	0.278	10	0.844	0.056	0.827
PM_{137}	0.302	5	0.748	0.116	0.702
PM_{204}	0.512	8	0.677	0.000	0.643
PM_{210}	0.284	9	0.816	0.023	0.792
PM_{238}	0.354	4	0.728	1.000	0.678
Mean	0.386	6.7	0.722	0.392	0.681

Table 5. Allele number, gene diversity, heterozygosity, and polymorphism detected by SSR markers in 38 *Arachis hypogaea* var. *vulgaris* accessions.

Marker	Major Allele Frequency	Allele No.	H_E (Expectedheterozygosity)	H_O (Observedheterozygosity)	PIC
PM_3	0.408	7	0.701	1.000	0.653
PM_{35}	0.351	7	0.741	0.054	0.700
PM_{36}	0.636	3	0.500	0.000	0.422
PM_{42}	0.532	4	0.602	0.613	0.532
PM_{45}	0.487	5	0.635	1.000	0.570
PM_{50}	0.536	6	0.661	0.036	0.630
PM_{137}	0.365	5	0.710	0.081	0.657
PM_{204}	0.632	4	0.500	0.000	0.419
PM_{210}	0.263	8	0.795	0.026	0.765
PM_{238}	0.382	5	0.700	0.971	0.648
Mean	0.459	5.4	0.654	0.378	0.600

Table 6. Allele number, gene diversity, heterozygosity, and polymorphism detected by SSR markers in 43 *Arachis hypogaea* var. *fastigiata* accessions.

Marker	Major Allele Frequency	Allele No.	H_E (Expectedheterozygosity)	H_O (Observedheterozygosity)	PIC
PM_3	0.279	9	0.784	0.977	0.750
PM_{35}	0.357	6	0.737	0.048	0.692
PM_{36}	0.600	3	0.501	0.000	0.401
PM_{42}	0.461	5	0.614	0.816	0.538
PM_{45}	0.500	4	0.619	1.000	0.547
PM_{50}	0.703	5	0.473	0.000	0.441
PM_{137}	0.452	5	0.681	0.048	0.630
PM_{204}	0.571	6	0.620	0.000	0.584
PM_{210}	0.810	6	0.336	0.000	0.324
PM_{238}	0.462	5	0.636	1.000	0.568
Mean	0.519	5.4	0.600	0.389	0.547

clustered with a similarity coefficient of 0.30. The latter clade clustered with a similarity coefficient of 0.32, which was joined to the two subclades with a similarity coefficient of 0.27 (var. *vulgaris*) and 0.31 (var. *fastigiata*). Four accesssions (Plant Introduction Number 030987, 147362, 172501, 172587) were outliers, which means they were not involved in any group. These results were a little different from the data obtained by sight. But, the plant type of most accessions were

consistent with phylogenetic dendrogram based on the SSR profiles. SSRs are the best marker system for this purpose since they can detect hypervariability in SSR motifs, which is usally selectively neutral (Amos *et al*., 1996, Ellegren *et al*., 1995, Varshney *et al*., 2005). The average expected heterozygosity (H_E) and observed heterozygosity (H_O) in accessions of peanut in our analysis were 0.713 and 0.387, respectively.

Tang *et al*. (2007) reported that the genetic diversity in cultivated peanut was much richer at the molecular level when compared with that in the morphological and biochemical levels. In this study, the molecular genetic diversity in var. *hypogaea* was the richest among those of three botanical types and each group had much more subclades. From this analysis, we could confirm that genetic diversity was so rich that the cultivated peanut could be identified into more botanical types as Krapovickas *et al*. (1994) did.

The results in the present study show each set from the three botanical varieties could be distinguished from each other with the SSR markers and can be further identified into different sub-groups (Tang *et al*., 2007). He *et al*. (2005) also reported that there were three groups of similar genome, in which botanical variety *fastigiata* with *vulgaris*, variety *peruviana* with *aequatoriana*, and variety *hypogaea* with *hirsute*. Since each group had the same pattern of bands using each of variety-specific markers, they postulated these specific markers might be actually related to these three groups. These results are mostly consistent with our results. The association between morphological traits and SSR loci of soybean landraces were also studied by Song *et al*. (1998) and the degree of association was meaningful. The cultivated peanut is an allotetraploid crop with A and B genomes. Less than four DNA fragments were amplified, because of the amplification of duplicated loci, each from the A and B genomes respectively, or fixed heterozygosity at a single locus, or possibly both of the above mechanisms (Hopkins *et al*., 1999). However, more than four DNA fragments were produced by certain SSR primers, which could not be interpreted this theory (Tang *et al*., 2007).

Better research on the relationship among the botanical varieties of *Arachis hypogaea* is essential prerequisite to be used effectively in the cultivated peanut germplasm management, and breeding programs and more marker and accessions need to be studied to understand the genetic diversity and the inter- and intra- variety genetic relationship for the future.

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