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SHORT COMMUNICATION

EST sequencing and SSR marker development from cultivated peanut (*Arachis hypogaea* L.)

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Keywords: *Arachis hypogaea* L., cultivars, EST-SSR, polymorphism, wild-type peanut.

Abbreviations: BLAST: Basic Local Alignment Search Tool
DAP: days after pegging
EST: expressed sequence tags
MAS: marker assisted selection
PCR: polymerase chain reaction
SSR: simple sequence repeat

Making use of the gene resources of wild type peanuts is a way to increase the genetic diversity of the cultivars. Marker assisted selection (MAS) could shorten the process of inter-specific hybridization and provide a possible way to remove the undesirable traits. However, the limited number of molecular markers available in peanut retarded its MAS process. We started a peanut ESTs (Expressed Sequence Tags) project aiming at cloning genes with agronomic importance and developing molecular markers. In this study we found 610 ESTs that contained one or more SSRs from 12,000 peanut ESTs. The most abundant SSRs in peanut are trinucleotides (66.3%) SSRs and followed by dinucleotide (28.8%) SSRs. AG/TC (10.7%) repeat was the most abundant and followed by CT/GA (9.0%), CTT/GAA (7.4%), and AAG/TTC (7.3%) repeats. Ninety-four SSR containing ESTs were randomly selected for primer design and synthesis, of which 33 pairs could generate good amplification and were used for polymorphism assessment. Results showed that polymorphism was very low in cultivars, while high level of polymorphism was revealed in wild type peanuts.

Peanut is one of the most important oil seed crops in the world, cultivated mainly in tropical, subtropical and warm temperate climates (Proite et al. 2007). The cultivated peanut is an allotetraploid (AABB, $2n = 4x = 40$), originated from chromosome duplication of hybrid between AA and BB wild type species, which happened about 3500 years ago. Polyploidy and self-pollination nature of this species blocked the genetic exchange between the cultivated and the wild type species which lead to limited genetic diversity of the major cultivars. Using of only very few central germplasms in breeding programs further limited the addition of genes with agronomic value. Due to its limited genetic variation peanuts are vulnerable to a wide variety of pathogens and abiotic stresses. For example, fungal foliar diseases of peanut such as rust, web blotch, and leaf spot cause severe loss of yield worldwide (Leal-Bertioli et al. 2009).

Wild type peanuts are genetically very diverse and selectively accumulated biotic and abiotic stress resistant genes during their adaptation to different harsh environments (Holbrook and Stalker, 2003). However, hybrids between the diploid wild type and the allotetraploid cultivated peanut are sterile (Tallury et al. 2005). In order to obtain tetraploid fertile offspring of the diploid wild type and the allotetraploid cultivated peanut, complicated breeding programs have to be designed. It normally takes many years to get a hybrid with agronomical value (Simpson et al. 1993; Stalker and Beute, 1993; Simpson and Starr, 2001). Marker assisted selection could increase the efficiency and predictability during the process of wild gene pool utilization. However, it was retarded by the small number of peanut molecular markers and the unavailability of high density linkage map (Burow et al. 2001; Moretzsohn et al. 2005). The attempt to develop more peanut molecular markers for construction of high density linkage map is of great importance. Microsatellites (SSRs) as DNA markers are highly polymorphic, highly abundant, show co-dominant inheritance, analytically simple and readily transferable between different plant species (He et al. 2003). Previous studies showed that SSR markers could detect more polymorphism in peanut than other molecular markers like RFLPs (Gimenes et al. 2007), AFLPs (He and Prakash, 2001; Gimenes et al. 2002) and RAPDs (Dwivedi et al. 2001; Subramanian et al. 2000).

Beside sequencing SSR enriched genomic library for SSR marker development (He et al. 2003; Han et al. 2006), searching SSRs using peanut EST information is an alternative way for marker discovery (Wang et al. 2006; Proite et al. 2007; Liang et al. 2009). We started an EST project using immature peanut seeds cDNA library of a Chinese cultivar to generate more sequence information for gene cloning (Li et al. 2009, Li et al. 2010) and marker development. The objectives of the present study were to develop more SSR markers from peanut using these EST sequences, and to detect the polymorphisms of the newly identified SSR markers in a collection of cultivated and wild type peanut accessions. The resulted SSR markers especially those showed polymorphism in different peanuts accessions would be valuable for peanut marker linkage map construction and marker assisted selection in the future.

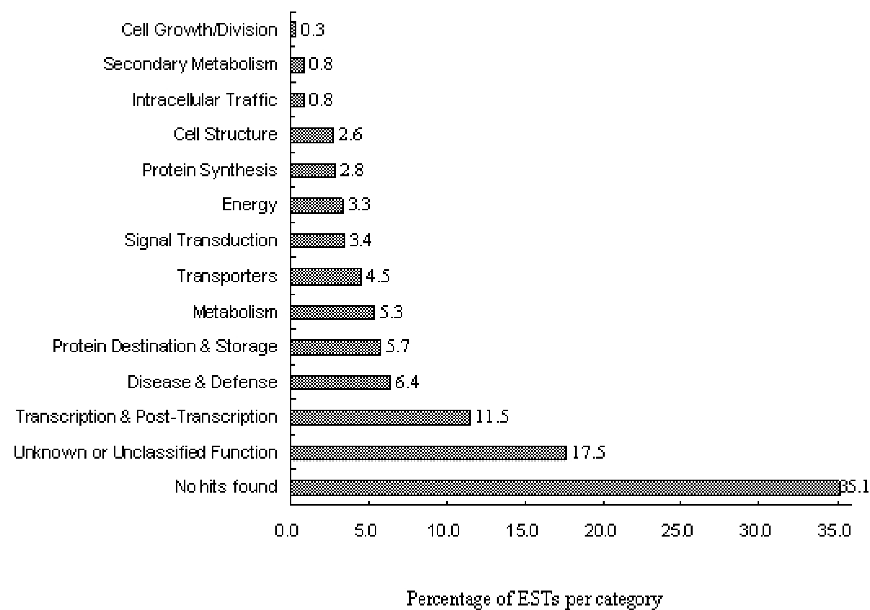

Figure 1. Percentage and functional categories of SSR containing ESTs.

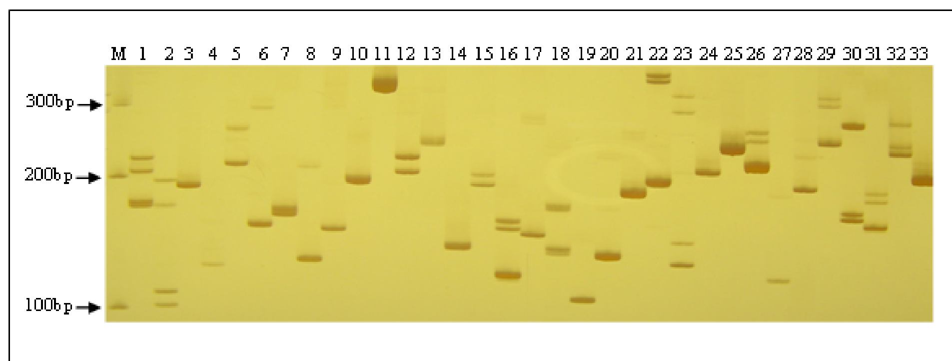

Figure 2. The amplification results of 33 pairs of primer using cultivated peanut Luhua-14. M, DNA marker; lanes 1 to 33, primer pairs of F2, F3, F9, F13; PD10, PD11, PD15, PD17, PD18, PD19, PD20, PD23, PD28, PD33, PD35, PD36, PD37, PD38, PD40, PD49, PD52, PD54, PD59; H2, H4, H6, H7, H8, H10, H11, H13, H14, H15.

MATERIALS AND METHODS

Plant material

Luhua-14 peanut used for cDNA library construction was obtained from Shandong Academy of Agricultural Sciences. A total of 98 accessions of wild type and cultivated peanuts were examined in this study. Twenty-five accessions belonging to section *Arachis* or *Heteranthae* which come from Nanning branch of the national wild peanut germplasm resource were used for polymorphism assessment. Seventy-three accessions belonging to cultivated species were used for genetic diversity analysis. Of these, fifty-six were collected from different provinces and the other 17 were from Wuhan national peanut germplasm resource belonging to *var. fastigiata*, *var. hirsuta*, *var. hypogaea* and *var. vulgaris* (Table 1 and Table 2).

Peanut cDNA library construction

The peanut seeds of different growing stages ranging from 20 to 60 DAP (days after pegging) were collected and placed into liquid N_2 immediately and stored in -80ºC freezer. RNA was extracted using RNAgent kit (Promega) according to the manufacturer's instructions. The quantity and quality of RNA was evaluated by spectrophotometry and agarose gel electrophoresis. cDNA synthesis and library construction followed the protocol of Stratagene's pBluescript II cDNA library construction kit.

Sequencing and ESTs analysis

Plasmid DNA was isolated from the randomly selected colonies and preserved in 96-well plate. Plasmid DNA was used as template for PCR amplification using T3 and T7 universal primers. PCR products were purified using AxyPrep™ PCR Cleanup Kit (AXYGEN), and sequenced using BigDye Terminator Cycle Sequencing Kit. Sequencing was performed on 3730XL Sequencer (ABI) using T3 or T7 primers. Sequences were edited by the software SEQENCHER and vector sequences were trimmed manually.

Analysis of microsatellites and primer design

EST sequences were searched for SSRs using SSR tool software (http://www.gramene.org/db/searches/ssrtool) to identify di- and trinucleotide and tetranucleotide SSR motifs. SSR motifs repeated more than five times in dinucleotide, four times in trinucleotide and tetranucleotide

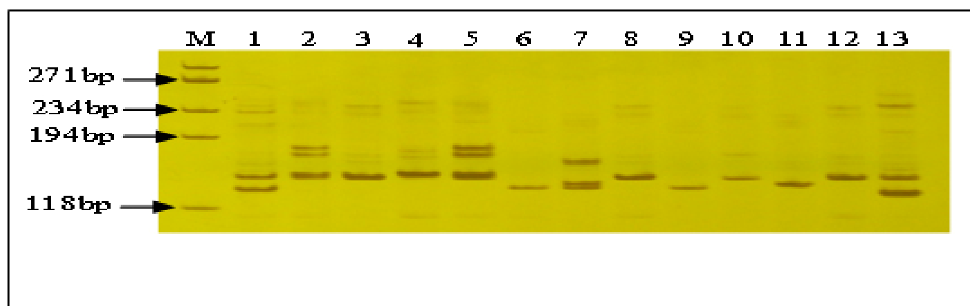

Figure 3. Polymorphism revealed by PD59 SSR maker in wild type peanuts. M, DNA Marker; lanes 1 to 12, wild type peanuts; lane 13, cultivated peanut Luhua-14.

Table 1. The accessions used for DNA polymorphism detection which came from Wuhan national peanut germplasm resource.

No.	Type	Cultivar	Origin
1	*var. fastigiata*	PI393531	America
2		Qiongshan Huasheng	China
3		Liaoning Silihong	China
4	*var. hirsuta*	Daye Pingtianzi	Jiang xi
5		Yingde Jidouzai	China
6		Jintang Shenwozi	Si chuan
7		Tuokexun Xiaohuasheng	China
8	*var. hypogaea*	Yangjiang Pudizhan	China
9		Xihua Goudou	China
10		Padou	China
11		Zhuzaidou	China
12		Yingde Zhusidou	China
13	*var. vulgaris*	Dedou	China
14		Bairizai	China
15		Guangliu	China
16		Sanyuening	China
17		Dingzi Xili	China

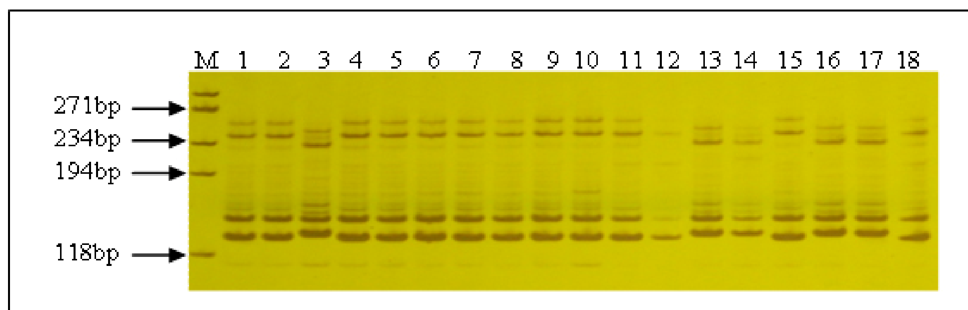

Figure 4. Polymorphism revealed by PD59 SSR maker in peanut cultivars. M, DNA Marker; lanes 1 to 18, cultivars PI393531, Qiongshan Huasheng, Liaoning Silihong, Daye Pingtianzi, Yingde Jidouzai, Jintang Shenwozi, Tuokexun Xiaohuasheng, Yangjiang Pudizhan, Xihua Goudou, Padou, Zhuzaidou, Yingde Zhusidou, Dedou, Bairizai, Guangliu, Sanyuening, Dingzi Xili, and Luhua-14.

Table 2. The accessions used for DNA polymorphism detection which collected from different provinces.

NO.	Cultivar	NO.	Cultivar	NO.	Cultivar
1	Huayu 16	20	Jinhua 1012	39	Yueyou 92
2	Huayu 17	21	Guihua 17	40	Trifumer
3	Huayu 19	22	Heyou 4	41	Hua 27
4	Huayu 21	23	Yueyou 551	42	Xuxi 1
5	Huayu 22	24	Quanhua 327	43	Xuhua 3
6	Huayu 23	25	Luhua 9	44	FT001
7	Luhua 10	26	Hua 17	45	Luhua 2
8	Luhua 11	27	AR-2	46	Luhua 12
9	Fenghua 1	28	Dayingzui	47	FT002
10	Fenghua 5	29	FT004	48	616
11	Zhufeng 1	30	Furonghuasheng	49	FT005
12	Qinglan 2	31	Yuhua 15	50	Haihua 1
13	Weihua 6	32	Quanhua 646	51	FT003
14	Shuangji 22	33	AR-3	52	Yueyou 116
15	Heihuasheng	34	GFA-1	53	FN006
16	Baisha 1016	35	Quanhua 10	54	Jihua 2
17	Minhua 5	36	Yuhua 14	55	Yuhua 10
18	Minhua 6	37	HS002	56	Luhua-14
19	Minhua 10	38	GFA-2		

were counted. Primers were designed using Primer 5 software.

DNA extraction and PCR amplification

Seventy-three cultivated peanuts and 25 wild type peanuts were used for polymorphism study. Genomic DNA was extracted from young leaf tissue using CTAB method (Murray and Thompson, 1980). DNA concentration was

determined by spectrophotometer. PCR mixture consisted of 1X PCR buffer, 200 µM of each dNTP, 1U Taq polymerase (TaKaRa) and 0.5 µM of each primer and 40-60 ng of template DNA. The thermocycling conditions started with 94ºC for 5 min, 10 cycles of 94ºC for 45 sec, XºC for 45 sec, 72ºC for 45 sec, and then 30 cycles of 94ºC for 45 sec, YºC for 45 sec, 72ºC for 45 sec followed by a terminal extension step of 72ºC for 10 min. The annealing temperatures (X and Y) were optimized for each primer to allow the best amplification (Table 3). Amplification products were visualized on 6.5% w/v denaturing polyacrylamide gels (19:1 acrylamide/bisacrylamide, 7 M urea) that were silver stained (Gao et al. 2009). Product size was estimated based on Φ×174-HaeIII digest DNA Marker (TaKaRa) or Bio DL100 (Bio Flux).

RESULTS AND DISCUSSION

Sequence analysis and SSR identification

A cDNA library was constructed using mRNA from immature seeds at different developmental stages of cultivated peanut Luhua-14. The insert length of the library ranged from 500-1500 bp with an average length of 900 bp based on PCR results for >1000 randomly picked colonies (data not shown). Sequences were edited by the software SEQENCHER and vector sequences were removed manually. Short sequences (<200 bp) and sequences with many unreadable nucleotides were discarded. 12,000 ESTs with good quality including the 7,456 ESTs deposited to GenBank (EE123340-EE127745; EG372473-EG374270; EG529454-EG530705) were subjected for SSR discovery. From these ESTs 610 SSR containing sequences were identified. A homology search against NCBI protein database was conducted by BLASTX with an E value of 0.02 as the cut-off threshold. Based on the BLAST results 610 SSR containing ESTs were grouped into 14 categories (Figure 1). More than half of these ESTs have no hits (35.1%) and corresponding unknown proteins (17.5%) in the database. The third largest functional category (11.5%) was transcription and post-transcription related factors, followed by disease and defense (6.4%), protein destination and storage (5.7%) and metabolism (5.3%) related genes.

Among 610 SSR-containing ESTs, 436 (71.5%) contained single SSR, 132 (21.6%) contained two SSRs, and the rest (6.9%) contained more than two SSRs. Among 841 SSRs identified, 66.3% were trinucleotides, followed by dinucleotides (28.8%), tetranucleotides (3.2%), pentanucleotides (0.8%), and hexanucleotides (0.8%). AG/TC was found to be the most common repeat motif (10.7%), followed by CT/GA (9.0%), CTT/GAA (7.4%), AAG/TTC (7.3%), AGA/TCT (6.2%), and AT/TA repeat motifs (5.4%). Previous surveys carried out on microsatellite abundance analysis in plant genomes have shown ATT and CTT were the most frequent trinucleotide repeat motifs (Lagercrantz et al. 1993; Ferguson et al. 2004), and AT was the most abundant dinucleotide repeat motif followed by AG/CT and GT/CA (Cuc et al. 2008; Han et al. 2006). He et al. (2003) found that GA/CT repeat was the most frequently dispersed microsatellite in peanut. Cuc and coworkers indicated that GT/CA repeat motif was the most common, accounting for 37.6% of all repeat types, followed by GA/CT repeat at 25.9% (Cuc et al. 2008). From this it can be concluded that SSR motifs are not equally abundant in eukaryotic genomes and the relative abundance of different motifs varies among species (Wang et al. 1994; Han et al. 2006).

Cultivated peanut Luhua-14was used to analyze the amplification quality of the identified SSRs. All 33 (35.1%) primer pairs with good amplification were selected for further analysis of polymorphism in different cultivars and wild type peanuts (Table 3, Figure 2).

Polymorphism study of wild type and cultivated peanuts

Twenty-five wild type peanuts were analyzed using the selected 33 SSR primer pairs. Most primer pairs could amplify distinct bands. However, some wild type peanuts failed to amplify any bands with some primer pairs. The possible reason for this result might due to the notable difference between wild type and cultivated peanuts.

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Jinhua 1012         ACCCTTTCACCCTCTTCAAATATCTCTCTTCTTCTTCTTCTTCTTCTTCTCAATCACCAATAAC
Liaoning Silihong   ACCCTTTCACCCTCTTCAAATATCTCTCTTCTTCTTCTTCTTCTTCTTCTCAATCACCAATAAC
Luhua-14            ACCCTTTCACCCTCTTCAAATATCTCTCTTCTTCTTCTTCTTCTTCT---CAATCACCAATAAC
PI393531            ACCCTTTCACCCTCTTCAAATATCTCTCTTCTTCTTCTTCTTCTTCT---CAATCACCAATAAC

Jinhua 1012         AAACAAACAAACAAAAATGCAATACTCGTACATGAGAAACTGTAGCAATAACAGAGCAGCCACATT
Liaoning Silihong   AAACAAACAAACAAAAATGCAATACTCGTACATGAGAAACTGTAGCAATAACAGAGCAGCCACATT
Luhua-14            AAACAAACAAACAAAAATGCAATACTCGTACATGAGAAACTGTAGCAATAACAGAGCAGCCACATT
PI393531            AAACAAACAAACAAAAATGCAATACTCGTACATGAGAAACTGTAGCAATAACAGAGCAGCCACATT
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Figure 5. Sequences comparison of polymorphic bands in different cultivars: Luhua-14, PI393531, Liaoning Silihong, Jinhua-1012.

Table 3. Peanut microsatellite markers with good amplification quality in Luhua- 14.

Primer pair	Forward primer (5'-3')	Reverse primer (5'-3')	Motif	Annealing		Expected Size (bp)
				X	Y	
F2	GGATTCATTCGAGGGTGG	AAGGCATCTTTGTCGTTG	(CTT)6	58	56	168
F3	CAGCAACTTTCTTCAACC	CTCTGAATCTCGCACCCT	(AGA)6	58	56	108
F9	TTCGAGCTTGAGGAAGAG	AACGGAATTTGAGATGATAG	(AATC)6	56	54	191
F13	ATCCTACGATTTCTTTACTG	TCGATGGAGGGATGATTT	(CT)25	50	48	152
H2	TCCCAGAACAACAATCAC	AGAAGAATACGAGCACCA	(TCT)5	54	52	215
H4	TCTTGATCTTATCTTCCCTG	CAAATGTGGTGTCGCTGA	(TCA)5	56	54	244
H6	CCAACTCGACTCGGCTTTC	TTGCTCCTCTAACAACCTTTCT	(TC)7	56	54	221
H7	CCTCACCAACCAGGATAC	TTTCGTCTTTGCAGTTTC	(CTT)4	54	52	122
H8	ATCAAGCCATAATATGTTCC	CAACCCAAGCACCTCTAC	(TA)6	54	52	193
H10	AGAGTAAGGGTGCTTCTG	ATGCTTCTCCCATTTCCA	(GAT)5	54	52	142
H11	CCGAACTCAACCCAAACA	AGCTCCGGCAAGAAAGAA	(CAC)4	56	54	178
H13	GCTTTTATTTCACATACATCAC	GGTAGGGCACTGTTCACTT	(CTT)7	54	52	165
H14	ACGAAGCCTAAGAACGGAAAG	ATGCCACCAGCAAAGGAG	(AC)5	56	54	258
H15	CGGTCGTAGTAGTTGTGG	GAGGCTTAAAGGAAGTGG	(CT)6	56	54	216
PD10	ACTTGCTTTGGATGCTTG	CTTTTCTTTTCCCGGACT	(TTA)7	50	48	208
PD11	CTCTTTCATTATTGGGTTTG	AGATTGTGCAGCCTCATC	(TAA)7	54	52	152
PD15	ACTAACACCATTTCCGACAG	TGGGAGCCTATGAAACCT	(TTC)5	58	56	163
PD17	CTTTCCCATATTCCTTCGT	TGTTCCTCCTCCTGCTTT	(TCT)6	56	54	129
PD18	ATCATCATCATCATCGTC	AGTTTCTGGTACTGTCGC	(TCA)6	54	52	152
PD19	CGTGGAGAACGTGCTTAG	CCATAGATCGAAATAGGTAG	(TTA)5	54	52	210
PD20	GAAGAAAATAGCAGGACT	CGTAGTATCTTGATAACCC	(CTT)6	56	54	254
PD23	TACAATGTTGCCTTTCAC	CAACTCCTCACTACTCCC	(TAA)6	56	54	205
PD28	ATTAAAGATGGCTTTGGAG	AGAGTAGTGGCAGTGACCT	(AAG)6	54	52	240
PD33	GGGGAACATGGTCTCATC	GGGCTTTCTCATAGGTGG	(TCT)5	60	58	139
PD35	GAACCTTAGAGCTTGTGG	GACGAGTAATTCTCCTTT	(TCA)5	50	48	194
PD36	GCATCTACCATTGGAGTG	ACAAAGCAAAGCGGATAC	(TAC)5	56	54	122
PD37	GTGAGAATGGAGACTTTG	TCACCATCACTTTATCAG	(TAA)5	54	52	149
PD38	GGGAGTGAAGTTAAAGAA	TGAATCCAACACCGTATC	(GCT)5	54	52	140
PD40	CTAGTACTTTTCTAAATTGGAC	TAATGGTTTTGGGTCACA	(GAA)5	54	52	108
PD49	CAACTGGTCCCAATAATCC	GCTATGCCTCCTTCTACAAAA	(ACA)5	58	56	135
PD52	CAGTTGAAATGCAAGGGTC	TGGAGTATCCAGGTGAGG	(AAC)5	58	56	189
PD54	CTCTTCTTCTGCCTCTGC	AGTAGCAACAACCCTCAT	(TCAA)5	56	54	202
PD59	ACCCTTTCACCCTCTTCA	AATGTGGCTGCTCTGTTA	(TCT)8	56	54	130

Initially 55 cultivated peanut genotypes were analyzed using the 33 primer pairs. Results showed that the polymorphism was very limited. Only one primer pair (PD59) could amplify polymorphic bands. In order to further evaluate the polymorphism of these 33 SSRs, seventeen cultivated peanut (from Wuhan national cultivated peanut germplasm resources) including *var. fastigiata, var. hirsuta, var. hypogaea and var. vulgaris*

were analyzed (Table 1). Similarly, low polymorphism was revealed by these SSR markers and only 4 (12.1%) primer pairs, F9, F13, PD20 and PD59, could amplify polymorphic bands (Figure 4).

For the 73 cultivated peanuts, primer pair PD59 could always amplify either one or both of two bands between 120 and 150 bp (Figure 4). The upper band showed same size within cultivated peanut accessions tested. However, the lower band displayed varied size in different cultivars (Figure 4). The lower band of four cultivated peanuts, PI393531, Liaoning Silihong, Jinhua-1012 and Luhua-14 were recovered from the agarose gel for sequence analysis. The sequencing results were shown in Figure 5. Cultivars PI393531 from America and Luhua-14 had one TCT less than Liaoning Silihong which belonging to *var. fastigiata* and Jinhua-1012 which belongs to *var. hypogaea*.

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