CELLULAR & MOLECULAR BIOLOGY LETTERS
Volume 9, (2004) pp 685 – 697
http://www.cmbl.org.pl

Received 17 March 2004
Accepted 17 September 2004

GENETIC DIVERSITY ANALYSIS IN VALENCIA PEANUT (*Arachis hypogaea* L.) USING MICROSATELLITE MARKERS

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Abstract: Cultivated peanut or groundnut (*Arachis hypogaea* L) is an important source of oil and protein. Considerable variation has been recorded for morphological, physiological and agronomic traits, whereas few molecular variations have been recorded for this crop. The identification and understanding of molecular genetic diversity in cultivated peanut types will help in effective genetic conservation along with efficient breeding programs in this crop. The New Mexico breeding program has embarked upon a program of improvement of Valencia peanut (belonging to the sub species *fastigiata*), because efforts to improve the yield potential are lacking due to lack of identified divergent exotic types. For the first time, this study has shown molecular diversity using microsatellite markers in the cultivated Valencia peanut (sub spp. *fastigiata*) from around the globe. In this investigation, 48 cultivated Valencia peanut genotypes have been selected and analyzed using 18 fluorescently labeled SSR (f-SSR) primer pairs. These primer pairs amplified 120 polymorphic loci among the genotypes screened and amplified from 3 to 19 alleles with an average of 6.9 allele per primer pair. The f-SSR marker data was further analyzed using cluster algorithms and principal component analysis. The results indicated that (1) considerable genetic variations were discovered among the analyzed genotypes; (2) The f-SSR based clustering could identify the putative pedigree types of the present Valencia types of diverse origins, and (3) The f-SSR in general is

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Abbreviations used: f-SSR - fluorescently labeled simple sequence repeat; SSR - simple sequence repeat; RFLP - restricted fragment length polymorphism; RAPD - random amplified polymorphism; AFLP - amplified fragment length polymorphism; MAS - marker assisted selection; DAF - DNA amplified fragment; UPGMA - unweighted pair-group method with arithmatic mean; PCA - principal component analysis; NTSYS - numerical taxonomy multivariate analysis system.

sufficient to obtain estimates of genetic divergence for the material in study. The results are being utilized in our breeding program for parental selection and linkage map construction.

Key Words: DNA, Genetic Diversity, Peanut, Simple Sequence Repeat

INTRODUCTION

The genus *Arachis* contains about 70 species all native to South America [1]. Nearly all *Arachis* species are diploid, but the cultivated peanut *Arachis hypogaea* (2n = 4x = 40) is tetraploid. Peanut is cultivated in 96 countries around the world and is an important legume crop [2]. Valencia peanuts belongs to the subspecies *fastigiata* and variety *fastigiata* occupying about 2% of the total area in United States of America. It is grown mostly in eastern New Mexico and west Texas occupying 10 to 12,000 ha. The major market for Valencia peanuts is in shell for human consumption.

Breeding for high yield and disease resistance requires selection of parents with wider genetic diversity. Considerable progress has been made in peanut improvement in the past few decades and has resulted in improved productivity all over the world. The productivity rate increased at 14.7 kg/ha in USA [3]. In the USA, the cultivar "Dixie Giant" is found in all pedigrees of runner market type peanuts and the small white "Spanish-1" cultivar in 90 percent or more pedigrees. These two lines contributed nearly 50% of the germplasm in runner cultivars [4].

A large number of methodologies are available for the characterization of germplasm variability, diversity, inter-relationship, as well as, individualization through macro molecular finger printing. Protein based technologies were limited in number and also environmentally influenced. DNA based technologies are the most reliable tools allowing for, not only, assessment of genetic variability, but also for individual DNA typing [5].

Some current DNA based methods are restriction fragment length polymorphism (RFLP) [6-10], random amplified polymorphic DNA (RAPD) [8, 11-13], amplified fragment length polymorphism (AFLP) [14,15] and simple sequence repeats (SSRs) [16, 17] markers, which were useful in classification of wild species but were not able to detect the polymorphism available in cultivated accessions. Microsatellites, also known as simple sequence repeats or SSRs, have been useful in detection of polymorphism among cultivated accessions of other species. These markers are small arrays of tandemly arranged bases (one to six) spread throughout the genomes and are abundant, informative, and co-dominant in nature. Microsatellites have proven to be an extremely valuable tool for genome mapping in many organisms [18, 19] and their applications span over different areas ranging from phylogenetic to forensic DNA studies, population genetics, and conservation management of biological resources and other uses [20, 21]. In plants, microsatellites have been recognized as useful molecular markers in marker assisted selection (MAS), analysis of genetic divergence.

Tab. 1. Details of peanut genotypes used in the present study for fluorescent-SSR based genetic diversity analysis.

S. No.	Variety/Accession number	Type/Name	Chromosome number	Country
1	New Mexico Valencia C	Valencia-NMSU	2n=40	USA
2	GT-101	Valencia PVP	2n=40	USA
3	GT-102	Valencia PVP	2n=40	USA
4	GT-136	Valencia PVP	2n=40	USA
5	Mc Ran	Valencia PVP	2n=40	USA
6	Sunland-1	Valencia PVP	2n=40	USA
7	Georgia Valencia	Valencia- Georgia	2n=40	USA
8	Georgia Red	Valencia-Georgia	2n=40	USA
9	PI 497288	Valencia	2n=40	Bolivia
10	PI 602105	Valentia Solonto	2n=40	Zimbabwe
11	PI 601293	Line	2n=40	Peru
12	PI 261989	RCM495	2n=40	Paraguay
13	PI 295173	Brownlong Mayne	2n=40	Israel
14	PI 259580	Valencia-8	2n=40	Jamaica
15	PI 408710	B1	2n=40	Uganda
16	PI 268517	Valencia ExSA	2n=40	Sudan
17	PI 259852	Valencia Potchef	2n=40	Malawi
18	PI 390383	W-C 960	2n=40	Colombia
19	PI 407679	Sanutsakorn 15	2n=40	Thailand
20	PI 320057	45	2n=40	Pakistan
21	PI 336942	Valencia	2n=40	Chile
22	PI 156668	Valencia	2n=40	Uruguay
23	PI 259601	Valencia	2n=40	Australia
24	PI 314980	UNIMK 433	2n=40	Russia
25	PI 315615	TATUI-55	2n=40	Japan
26	PI 338336	Valencia	2n=40	Venezuela
27	Sadovo-3211	Advanced Line	2n=40	Bulgaria
28	Sadovo-3190-5	Advanced Line	2n=40	Bulgaria
29	Sadovo-3256-V	Advanced Line	2n=40	Bulgaria
30	Sadovo-3240	Advanced Line	2n=40	Bulgaria
31	Sadovo-3663	Advanced Line	2n=40	Bulgaria
32	Sadovo-3542	Advanced Line	2n=40	Bulgaria
33	Sadovo-3190-b	Advanced Line	2n=40	Bulgaria
34	Sadovo-3840	Advanced Line	2n=40	Bulgaria
35	Sadovo-36119	Advanced Line	2n=40	Bulgaria
36	Kalina	Local Variety	2n=40	Bulgaria
37	Rossita	Local Variety	2n=40	Bulgaria
38	Sadovo-3103-a/107	Advanced Line	2n=40	Bulgaria
39	Sadovo-3180-2/184	Advanced Line	2n=40	Bulgaria
40	Sadovo-3303-3/189	Advanced Line	2n=40	Bulgaria
41	Sadovo-3646/229	Advanced Line	2n=40	Bulgaria
42	Sadovo-3652-1/297	Advanced Line	2n=40	Bulgaria
43	Sadovo-3654-1/308	Advanced Line	2n=40	Bulgaria
44	Sadovo-3667/234	Advanced Line	2n=40	Bulgaria
45	Sadovo-3805/258	Advanced Line	2n=40	Bulgaria
46	Sadovo-3873/317	Advanced Line	2n=40	Bulgaria
47	Sadovo-3881/294	Advanced Line	2n=40	Bulgaria
48	Sadovo-3897/MCO	Advanced Line	2n=40	Bulgaria

In cultivated peanut, RFLP, RAPD and AFLP markers have identified little variation [6] or exotic lines [8]. No polymorphism between *A. hypogaea* and *A. monticola* was observed by [22], while a significant amount of variations have been reported among *Arachis* species [6, 7] and between botanical varieties of cultivated peanut using AFLP and DAF technique [14]. AFLP, using restriction enzyme combination *Mlu*I/*Mse*I, could detect polymorphism in closely related cultivated peanut genotypes [15]. Six SSRs detected polymorphism in cultivated peanuts out of 66 SSR loci [23]. This investigation was carried out to identify genetic diversity in Valencia type peanuts belonging to the subspecies *fastigiata* variety *fastigiata* using the fluorescence-based SSR (f-SSR).

MATERIAL AND METHODS

Plant materials

Plant materials consisted of 48 Valencia peanut genotypes from 20 different countries around the world (Tab. 1). These Valencia germplasm were obtained from the United States Department of Agriculture, Plant Genetic Resource Conservation Unit, Griffin, Georgia.

DNA extraction

Genomic DNA was isolated from green leaves of 25-day-old seedlings. Each sample consisted of leaves pooled from 3-5 seedlings (approximately 2g), and was processed as per [24] until the crude DNA precipitation step. The DNA was treated with 20µg/ml RNase and incubated at 37^{o}C for 30 min. DNA was extracted with Chloroform: Isoamyl alcohol (24:1 v/v), and re-precipitated with two volumes of absolute ethanol. The purified DNA was dissolved in TE (10 mM Tris, 0.1mM EDTA, pH 8.0) buffer and quantity was determined using a Flurometer.

SSR analysis

Initially thirty-two geographically divergent genotypes were analyzed using six unlabeled SSR primer pairs [23] and 19 primers from [16]. Samples were analyzed using an agarose mixture (1:1 ratio w/w) of Metaphor (FMC corp. USA) and Agarose and those primer pairs showing polymorphism were fluorescently labeled and later analyzed for all 48 genotypes (Tab. 2). PCR reactions were prepared and amplified in a PE 9700 thermocycler. Each 10µl PCR reaction consisted of 1X PCR buffer, 0.4 mM dNTP, 2.5 mM $MgCl_2$, 0.1 U *Taq* Polymerase (Ampli Taq Gold, ABI) and 1.5 Pico moles of each primer and 10ng of template genomic DNA. The amplification conditions had an initial hot start of 95^{o}C for 12 min, 45 cycles of 94^{o}C for 15 sec, 55^{o}C for 30 sec, 72^{o} C for 60 sec followed by a terminal extension step for 72^{o}C for 7 min.

For sequencer band detection, 2µ l of the amplified DNA was mixed with 0.3µL of Gene Scan 350XL ROX standard (Applied Biosystems, Foster City, CA) except for amplified products of primer 4-24 wherein Gene Scan 500XL ROX standard (Applied Biosystems, Foster City, CA) and 1µL of 50% formaldehyde

loading buffer and sample were denatured at 95°C for 3 min. before loading. The samples were loaded and electrophoresed on 12 cm long 5% w/v denaturing long ranger FMC gels on an ABI 377 sequencer (Applied Biosystem, Foster City, CA). Data were collected by the Gene Scan software (Applied Biosystem) and later gels were photographed.

Tab. 2. Details of the polymorphic primers used in microsatellite analysis.

Sl. No	Primer pair	Primer sequence (5'-3')	No. of poly-morphic alleles	Size range of alleles (bp)	Average number of bands per genotype	Poly-morphism percent (%)
1	Ah 4-4	CGATTTCTTTACTGAGTGAG ATTTTTTTGCTCCACACA	4	92-100	1.00	100
2	Ah 4-20	ACCAAATAGGAGAGAGGGTTCT CTCTCTTGCTGGTTCTTTATTAACTC	7	200-223	2.00	100
3	Ah 4-24	TTCTGATTTTAGTAGTCTTCTTTCACT CTCCTTAGCCACGGTTTCT	3	416-421	1.02	100
4	Ah 4-26	TGGAATCTATTGCTCATCGGCTCTG CTCACCCATCATCATCGTCACATT	11	150-213	3.02	100
5	Ah 6-125	TCGTGTTCCCGATTGCC GCTTTGAACATGAACATGCC	2	190-196	1.06	66.6
6	Lec1	CAAGCATCAACAACAACGA GTCCGACCACATACAAGAGTT	19	218-270	1.68	100
7	PM-3	GAAAGAAATTATACACTCCAATTATGC CGGCATGACAGCTCTATGTT	9	188-221	2.65	100
8	PM-15	CCTTTTCTAACACATTCACACATGA GGCTCCCTTCGATGATGAC	4	177-181	1.90	100
9	PM-32	AGTGTTGGGTGTGAAAGTGG GGGACTGGGAACAGTGTTTATC	6	90-109	1.30	100
10	PM-35	TGTGAAACCAAATCACTTTCATTC TGGTGAAAAGAAAGGGGAAA	8	95-141	2.90	100
11	PM-36	ACTCGCCATAGCCAACAAAC CATTCCCACAACTCCCACAT	8	160-220	1.18	100
12	PM-50	CAATTCATGATAGTATTTTATTGGACA CTTTCTCCTCCCCAATTTGA	7	99-114	1.04	100
13	PM-53	CCTATCCTATGGGTCACTAGCC GCTTGTGCTCATCTTGAGTTTT	3	116-120	1.04	100
14	PM-73	AGTGTTGGGTGTGAAAGTGG GGGACTCGGAACAGTGTTTATC	3	100-112	1.23	100
15	PM-179	CTGATGCATGTTTAGCACACTT TGAGTTGTGACGGCTTGTGT	5	82-107	2.98	100
16	PM-183	TTCTAATGAAAACCGACAAGTTT CGTGCCAATAGAGTTTTATACGG	9	101-139	1.00	100
17	PM-200	GCTATGTGGGAAAAAATACTGCTT CAGATGTGTGTGTGTGTGTG	3	140-160	2.69	100
18	PM-210	CCGCAGATCTTCTCCTGTGT CCTCCTCATCCTCTAAACTCTGC	8	179-211	1.35	100

Data analysis

The electrophoretograms in the range of 75 to 350 or 500 bp (for primer pair Ah 4-4) were analyzed and compared using Gene Scan software, (Perkin Elmer Applied Biosystems). Each fragment size was treated as a unit character for analysis and converted to binary code (1/0 = +/-). The complete data pooled over

all the primer pairs in each case, the 1/0 matrix was used to calculate the genetic (dis)similarities using Jaccard coefficient [25].
The resulting matrices from the pooled data set were utilized to ascertain the genetic interrelationship by: (a) constructing phenetic trees using unweighted pair-group method with arithmatic mean (UPGMA) cluster analysis to infer the phenetic relationships; (b) partitioning the variance of the datasets using principal component analysis (PCA) and plotting the first two principal components. The distance matrix and dendogram were constructed with the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) version 2.1 software package (Exeter Software, Setauket, NY) software package [26].

RESULTS AND DISCUSSION

The assessment of genetic diversity or similarity is not only important for crop improvement efforts but also for efficient management and protection of germplasm resources. This information can be obtained through DNA fingerprinting approaches capable of exhibiting large number of loci for extensive variability. Valencia peanut collected from diverse origins was analyzed using a highly repeatable PCR based fingerprinting assay known as Simple Sequence Repeats or microsatellite markers.

SSR fragment size range and polymorphism in accessions

Initial evaluation of thirty-two accessions of diverse origin belonging to the Valencia peanut was performed using 19 unlabelled primer pairs from [16] and six from [23]. The amplified products were analyzed for polymorphism using agarose gels (Fig. 1) and the results revealed 18 primer pairs detecting polymorphism among the genotypes screened. These 18 informative primers were fluorescently labeled and used to analyze all the 48 Valencia genotypes.

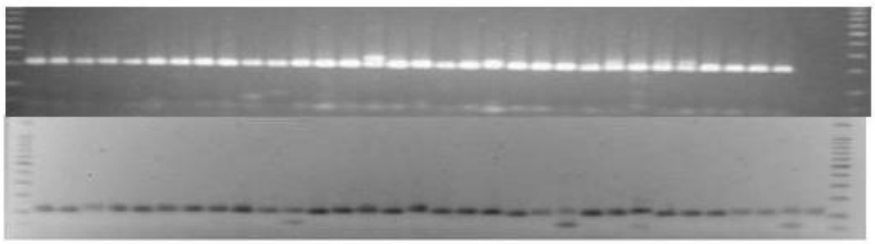

Fig. 1. Two primers detecting polymorphism using unlabeled SSR primer-pairs on metaphor agarose gels.

These 18 primer pairs amplified 120 different loci. A total of 119 polymorphic loci were recorded from the genotypes screened. The capacity of different primer pairs to amplify microsatellite loci ranged from 3 to 19 alleles (average of 6.72). The number of bands ranged between 27 to 35 (average of 31.2) among genotypes for all primer pairs, while the average number of bands per genotype

per primer pair ranged between 1.00 to 3.02 (Tab. 2). The mean number of fragments per accession and range of fragments over genotypes did not vary much indicating the desirable attribute for their utility in diversity analysis. Such data reveals a near-uniform selection pressure over all the genotypes during development and cultivation. Variable number of band amplification between improved cultivars and land races of Cassava was reported [27]. In peanut the frequency of SSR band/allele ranged from 0.020 (i.e. present only in one genotype) to 1 (present in all). Levels of polymorphism per primer were very high 100% in most primer pairs except 6-125, which exhibited only 66.6% polymorphism among the genotypes screened. All the primer pairs were informative, among which primer Lec1 amplified 19 alleles. Primer pair Lec1 amplifies an $(AT)_n$ SSR locus with in the 3'untranslated region of the lectin gene [28].

The present study, which consisted of 119 polymorphic markers, may have provided genome coverage for reliable molecular diversity analysis. Accordingly, the genetic groupings inferred from the present data were supported by the high boot strap and cophenetic values indicating the robustness of the data (data not presented).

Diversity among accessions

The similarity matrix using Jaccard similarity [29] coefficient revealed the highest similarity coefficient (0.91) between genotypes of Bulgarian origin, while the lowest similarity coefficient of 0.16 was detected between PI 497288 of Bolivia and S-3663 of Bulgaria, the average similarity among accessions was 0.45 (data not shown).

The cluster analysis using UPGMA based on similarity coefficients was done to resolve the close genotypic relationships between the analyzed Valencia peanut genotypes (Fig. 2). Three clusters could be seen at the 40% Jaccard similarity coefficient level. The first cluster consisted of four sub-groups A, B, C and D with 6, 9, 2 and 1 genotypes respectively, and this cluster consisted mainly of genotypes from different continents excluding Europe. The sub-group A of the cluster consisted of North American Valencia types because almost all U.S. peanut cultivars can trace their ancestry to a narrow genetic base of plant introductions [30]. The sub- group B consisted mostly of South America, Central America and Africa, which are primary and secondary centers of diversity. The sub-group C and D consisted of genotypes from Columbia, Venezuela and Japan. The second cluster consists of three E, F and G sub-groups of 2, 21 and 1 genotypes respectively; the F and G sub-groups consisted of all the Bulgarian genotypes. The Bulgarian group was similar; 53 percent had two major clade, the first consisting of 5 genotypes that were derived from a cross between a Valencia type and Shulamith (PI 372572 - Virginia bunch type). The second sub-group consisted of genotypes mostly derived from Valencia X Valencia crosses, with the female parent having ancestral record of a Virginia bunch type from Israel. The Variety Rossita derived from S2609 (Valencia) X Shulamith [31] was grouped apart from other Bulgarian Valencia genotypes. The sub-group

G containing genotype S 3190-b grouped independently to that of the other Bulgarian type; this genotype had the parentage of Rossita but may have been selected more towards the Virginia type. The cluster 3 consisted of 6 accessions from diverse origins, in which the American Valencia type, Georgia Red, and Georgia Valencia clustered with other Valencia types at 33-34% similarity coefficient levels; this may be due to the presence of runner type (Florunner in Georgia Red and UF 85179 in Georgia Valencia) ancestral parentages in these two genotypes [32, 33].

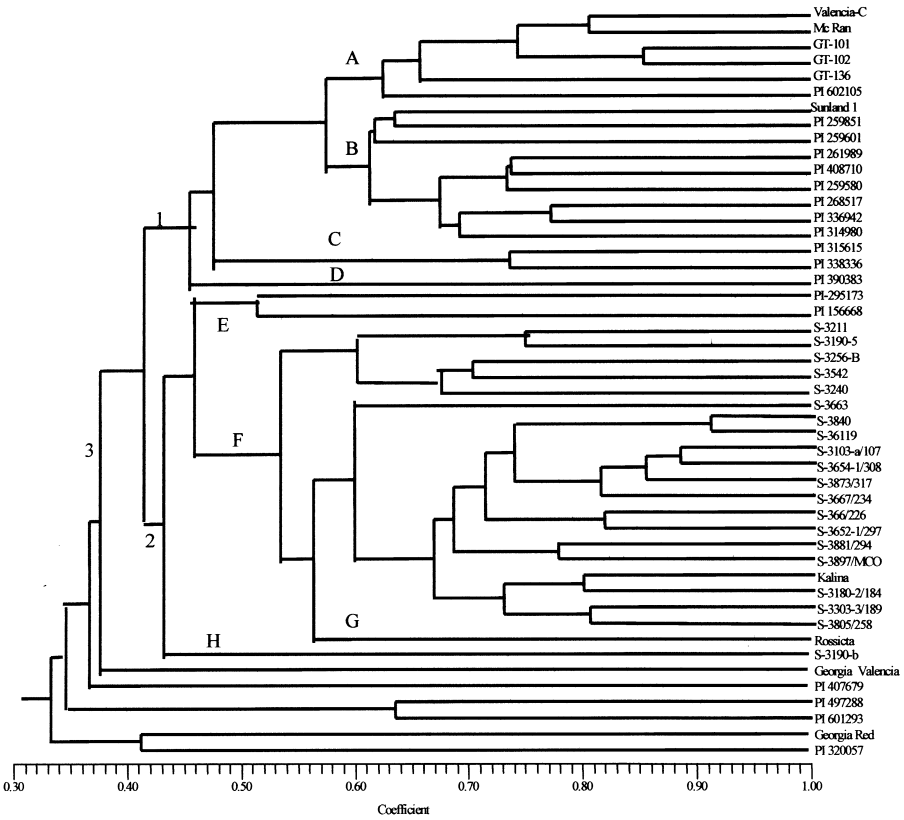

Fig. 2. UPGMA clustering of 48 peanut genotypes based on 120 SSR markers amplified by f-SSR primer pairs.

Thus, a cross between Valencia and Virginia (runner types) and selecting for the desired Valencia types can increase the variability among the cultivated Valencia types, thus paving the way for further breeding programs to maximize diversity.

Principal component analysis

The PCA is one of the multivariate approaches of grouping based on similarity coefficients or variance–covariance values of the component traits of the entries.

This gives more information about major groups, while a cluster analysis provides higher resolution among closely related population [34]. In our study, 86% of the variations in the estimates of genetic similarity was explained by the first three components indicating the suitability of microsatellite based marker approaches for genetic clustering. The first principal component explained approximately 80% of the variation and resolved the Valencia genotypes based on subspecies parental history (that is, whether both parents were Valencia X Valencia or one was runner or Virginia type) and the geographical isolation. The second component explained approximately 4% variation of the data.

A total of four distinct clusters were revealed by the first two principal components (Fig. 3). Cluster-I includes the true Valencia types of USA along with the genotypes from Africa, Australia, and Russian origin. The genotype of New Mexico- Valencia C and genotypes from H & W Genetex Inc were in the same group, indicating that the genotypes in the group were in the selection pressure for true Valencia types.

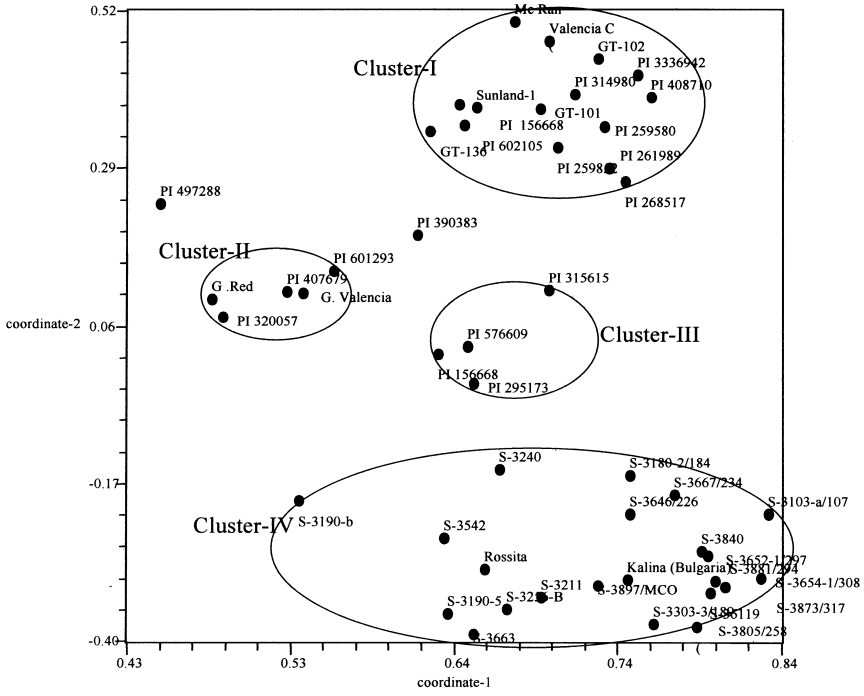

Fig. 3. Two-dimensional plot of genetic diversity among the 48 peanut genotypes as revealed by the PCA based on microsatellite markers obtained with eighteen primer-pairs.

The cluster-II consisted of Georgia Red and Georgia Valencia grouped along with genotypes from Pakistan, Peru, and Thailand. Georgia Red has been derived for a cross between the component lines Florunner and New Mexico Valencia A through mass and pedigree selection [32]. Georgia Valencia was

developed by crossing Georgia Red X UF85179 by pedigree selection within F_2, F_3 and F_4 segregating populations [33]. Thus, these runner genotypes would have grouped differently from other American Valencia types.

The cluster-III consisted of genotypes from Venezuela, Uruguay, Israel and Japan. These lines were grouped separately from other groups and had parents mainly from runner or Valencia types. The cluster-IV consisted mainly of genotypes from Bulgaria exhibiting the distinctiveness of the Bulgarian genotypes for the geographical region. This may be due to the involvement of a Virginia bunch type ancestral parent in most of the present genotypes. The present study consisted of 22 genotypes from Bulgaria, which were mostly derived from eight parents [31], thus this narrow genetic base could have resulted in the isolation of Bulgarian genotypes.

The present analysis could clearly distinguish bold seeded and high yielding Bulgarian Valencia peanut genotypes from the rest of the genotypes; thus it is evident that crossings between different types increases the molecular diversity and provides more opportunity for breeding programs. In conclusion, the present investigation demonstrates the potential of SSR markers to revealing extensive genetic polymorphism useful for genetic diversity analysis and genotyping individuals. Thus microsatellites have revealed that US germplasm are not that narrow and also the European germplasm from Bulgaria are unique that they form a separate group. Thus this information is presently used in development of molecular maps and markers to assist in efficient breeding of various traits, such as yield, flavor, oil quality, resistance to pest and diseases, and other economically important traits.

Peanut (*Arachis hypogaea*) breeding programs with a goal of rapid cultivar development have used mainly elite breeding lines and cultivars, which has resulted in development of breeding materials with a narrow genetic base. Utilization of exotic germplasm resources in breeding programs is needed to enhance the diversity of breeding populations for selection gains in the future.

While selecting the germplasm for any breeding program, it is important to consider genetic background of the line. The less divergent and more adaptive lines are those that show additive gene effects [35]. When diversity between parents increases, dominance effects and epistatic variances have significant role in inheritance of quantitative traits [8]. The main reason for not using the exotic germplasm in the breeding program is due to the fact that exotic germplasm has little to offer that the time and efforts for breeding can't be justified. The improvement is normally aimed at rapid and short-term cultivar development rather than long term germplasm development using exotic germplasm [8].

In the study we observed that by adding fluorescent labeling to the primers and semi-automated detection technology, the study had a significant advantage in allele detection and type of alleles compared to earlier experiences with radioactive SSR and Silver stained gels. The present technique was an improvement over original methods by increasing the scoring and typing efficiency with reduced risk of exposure to the radioisotope or stains of silver ions.

Acknowledgments. The authors wish to thank Dr. Guohao He for furnishing SSR primer information from his research. Thanks to Drs Hari D. Upadhyaya and H. Budak for useful comments on the manuscript. This research was funded in part by grants from the USAID Grant # LAG-G-00-96-90013-00 to the Peanut Collaborative Research Support Program through University of Georgia and New Mexico Agricultural Experiment Station. Dr. G. K. Krishna would like to thank Mahyco Research Foundation, Hyderabad, India for providing sabbatical leave to conduct this study.

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