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GENOMIC FINGERPRINTS OF *ARACHIS HYPOGAEA* L. NATURAL GERMPLASM AS REVEALED BY iPBS MARKERS

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ABSTRACT

Arachis hypogaea, L. is an oil seed crop with a worldwide importance and the seeds are eaten at one of several stages from immature to fully ripe, raw, or cooked. The aim of the study was to analyze specific iPBS fingerprints of twenty-one accessions of peanut that were collected in the places of their natural occurrence in Bolivia and describe the existing genetic polymorphism. For genomic imprinting, three different iPBS markers were chosen - 1882, 2079, 2274 and a PCR reactions were performed. Obtained iPBS fingerprints were evaluated for the presence/absence of individual amplified loci and scored in 1/0 matrices. A Jaccard coefficient of genetic similarity was applied in UPGMA analysis for dendrogram construction. Polymorphism level was achieved in the range from 48% up to the 75% per primer. None of the iPBS markers used in the study was considered to distinguish all of the analyzed peanut accessions, but combining them in the final analysis, the level of genomic polymorphism was sufficient to clear separating of iPBS fingerprints of the collected accessions and unique iPBS loci were recorded in genomes of some of them. We found that, by selecting the appropriate iPBS markers, it is possible to characterize the peanut genome in the individual level with a specific fingerprint.

Keywords: *Arachis hypogaea*, L.; iPBS markers; fingerprints; germplasm

INTRODUCTION

Arachis hypogaea, L. is a world known oil seed crop that is harvested mainly in semi-arid tropical, subtropical and temperate regions ***(Naidu et al., 1999; Proite et al., 2007)***. The earliest archeological reports of peanut comes from Peru and dates back to 3900–3750 years ago ***(Hammons, 1994)***. The genus *Arachis* originated in the southwestern part of Mato Grosso do Sul, Brazil, or northeast Paraguay ***(Simpson et al., 2001)***. *Arachis* contains 81 described species that have been classified in nine distinct taxonomic sections based on cross-compatibility, morphological characters, and geographic origin ***(Krapovickas and Gregory, 1994; Valls and Simpson, 2005)***.The cultivated form of peanut originated in the area of southern Bolivia to the northwestern Argentina, where the great range of ecologically distinctive environments exist in the Andes eastern foothills ***(Stalker and Simpson, 1995)***. The diversity of uses of peanuts is an evidence of its antiquity - the seeds are eaten at one of several stages from immature to fully ripe, raw, or cooked. They are processing by boiling, broiling, roasting, crushing or grounding and mixing with other food.

The whole young pods are occasionally used in soups after boiling, peanuts are further used to make a beer and a nonalcoholic drink. The oil is also processed into soap ***(Stalker and Wilson, 2016)***. *Arachis hypogaea* is an allotetraploid species (2n = 4x = 40, AABB) with a very large and complex genome. Cytological, it behaves mostly as a diploid, but multivalents can result in skewed genetic ratios and likely account for many of the "off types" ***(Leal-Bertioli et al., 2015)***.

Because of economic importance of cultivated peanut, its germplasm is preserved and maintained around the world in different *ex situ* collections ***(Benz, 2012)***. The largest collections of *Arachis* germplasm are in India (International Crops Research Institute for the Semi-Arid Tropics), United States (United States Department of Agriculture), China (Oil Crops Research Institute, Chinese Academy of Agricultural Sciences), and Brazil (Empresa Brasileira de Pesquisa Agropecuaria), and smaller collections do exist in many countries around the world ***(Richards and Volk, 2010)***.

Many different techniques are applied to describe and characterize plant genetic resources. One of the most modern are those based on DNA markers. Different specific regions of plant genomes are used as DNA markers and many of different techniques were applied successfully for a wide range of plants, such as SCoT markers for cultivated castor ***(Vivodík et al., 2019)***, SSR markers for triticale ***(Balážová et al., 2016)***, RAPD markers for wheat ***(Kuťka-Hlozáková et al., 2016)***, or iPBS markers for common ivy ***(Žiarovská et al., 2019)***. In the case of peanut, DNA markers were used to analyse different questions connected to its genome variability. Microsatellite markers were applied to analyse cultivated peanuts by ***He et al. (2005)*** and test its transferability to analyze peanuts by ***Gimenes et al. (2007)***, specific SSR markers were isolated ***(Cuc et al., 2008)*** and developed from the EST sequencing by ***Song et al. (2010)***, intron sequences and microsatellite markers were used for the purpose of molecular breeding ***(Pandey et al., 2012)*** and study of phylogenetic relationships of peanut by ***Moretzsohn et al. (2013)***. Amplified fragment length polymorphism (AFLP) markers were applied in the studies of identification of polymorphic regions of peanut genomes by ***He and Prakash (1997, 2001)***. Random amplified polymorphism detection (RAPD) fingerprints were defined for *Arachis hypogaea*, L. genome by ***Raina et al. (2001)*** and used to link some important resistance genes ***(Mondal et al., 2007)***.

Here, iPBS markers were used to analyze the genome diversity of natural accessions of peanut. Up to now, this markers were not used for the analysis of *Arachis hypogaea*, L.. iPBS markers were developed based on the specific sequential characteristics of primer binding sites typical for retrotransposon elements by ***Kalendar et al. (2010)***. Retrotransposons are an abundant and natural part of plant genomes and have some specificities that allow to use them

as universal marker systems without a previous knowledge about a very concrete retrotransposons that are presented in the genome of a specie of interest. Moreover, retrotransposons are well known as markers revealing insertion fingerprints based on different biotic or abiotic stress history of plant genomes ***(Kalendar et al., 2010)***, what makes them a very good marker system when analyzing natural plant populations. This marker technique provide repeatable, time efficient and universal approach, that can be used for fingerprint profiling. They were used in genome polymorphism analysis in many plant species such as *Cicer* spp. ***(Andeden et al., 2013)***, *Adonis* spp., *Paeonia anomala* L., *Adenophora lilifolia* (L.)A.DC. and *Digitalis grandifolia* Mill. ***(Boronnikova and Kalendar, 2010)***, *Fagaceae* species ***(Coutinho et al., 2018)***, *Vitis vinifera* L. ***(Gou et al., 2014; Milovanov et al., 2019)***. They are also applied in high-throughput sequencing for the development of new types of retrotransposon-based markers ***(Monden et al., 2014)***.

The objective of our study was to analyze specific iPBS fingerprints of twenty-one accessions of *Arachis hypoaea*, L. that were collected in the places of their natural occurrence in Bolivia and describe the existing genetic polymorphism.

MATERIAL AND METHODS

Plant material

Twenty one different accessions of peanut germplasm seeds have been taken in Chuquisaca Department, Bolivia as original plant sources. The seeds were transferred to the Faculty of Tropical Agriculture, Czech University of Life Sciences in Prague; Czech Republic and planted in pots. Young plants were transferred to the Research center AgroBioTech; Slovak University of Agriculture in Nitra, Slovak Republic where biological material was processed and stored at -50 ° C until DNA extraction.

Total genomic DNA extraction

Total genomic DNA was extracted from collected leaves (5 for each plant) using a GeneJET Plant Genomic DNA Purification Mini Kit ThermoScientific) and following the manufacturer´s instructions. Concentration and purity of extracted DNA was measured by NanoPhotometer® P-Class by Implen and checked in 1% agarose gel electrophoresis.

PCR and iPBS reaction performing

DNA from all samples was mixed firstly by 1 µL per accessions and the resulted solution was diluted to three concentrations: 1:10, 1:25 and 1:50. Mixed DNAs was used in the polymerase chain reaction with 10 randomly selected iPBS primers (1830, 1845, 1846, 1881, 1882, 1886, 1897, 2079, 2274 and 2392) ***(Kalendar et al., 2010)*** and based on the screening, three of them were used further in the analysis. It was conducted in 10 µL volume. Amplification were performed by Agilent Technologies SureCycler 8800 using 2X EliZyme HS Robust MIX (MasterMix). PCR program setup: initial denaturation 95 ° C for 3 min, followed by 35 cycles of 95 ° C for 30 s, 55 ° C for 40 s, 72 ° C for 2 min and final extension at 72 ° C for 5 min. Based on the preliminary results, primers 1882, 2079, 2274 were selected (1:25 DNA dilution) for iPBS analysis of peanut varieties. The same PCR mix and PCR program were used to amplify iPBS profiles.

Data processing

Electrophoresis was performed in 3% agarose gel stained with GelRed in 1X TBE buffer for initial screening PCR reactions. Samples were stained with GelRed (Biotia), loaded on gel, and photographed under ultraviolet light by UV and white light transilluminator UVstar, Biometra, wavelength 312 nm. For iPBS amplicons processing, electrophoresis was done on 10% PAGE in a 5X TBE buffer. The gel was stained in 5X TBE mixed with GelRed for 1 hour and photographed under ultraviolet light by UV and white light transilluminator UVstar, Biometra, wavelength 312 nm.

Obtained iPBS fingerprints were evaluated for the presence/absence of individual loci and scored in 1/0 matrices. A Jaccard coefficient of genetic similarity ***(Jaccard, 1908)*** was applied in UPGMA analysis for dendrograms construction. Cophenetic coefficients ***(Lessig, 1972)*** were calculated for dendrograms and polymorphic information content coefficients (PIC) ***(Weber, 1990)*** for individual iPBS primers used in the study. Dendrograms were constructed by SYNTAX software.

RESULTS AND DISCUSSION

The iPBS markers has been proved to be a powerful DNA fingerprinting technology. This method is used for investigation of new long terminal repeats type of retrotransposons in any eukaryotic organism. It was reported that primers that are designed to hybridize the conserved regions of the primer binding sequences of retrotransposons, are very efective in PCR amplification ***(Fang-Yong and Ji-Hong 2014; Kalendar et al. 2019)***. This method can differentiated close genotypes ***(Antonius-Klemola et al., 2006)*** and it can be used without prior genome sequence knowledge, is highly reproducible and has high stringency ***(Guo et al. 2014; Antonius-Klemola et al. 2006)***. This method can be applied not only to endogenous retroviruses, but also for both the *Gypsy* and *Copia* LTR retrotransposons, too ***(Melnikova et al. 2012)***.

Inter primer binding sites polymorphism was analyzed in the study for the peanut accessions. All of the iPBS markers that were used in the study, generated a sufficient amount of amplicons, but the variability in achieved percentage of polymorphism **(table 1)**.

Table 1 Characteristics of the iPBS fingerprints used for peanut germplasm analysis

iPBS marker	Number of fragments	% of polymorphism	PIC	Cophenetic coefficient in UPGMA
1882	70	48	0.43	0.89
2079	168	67	0.46	0.83
2274	205	75	0.33	0.91

Amplification of retrotransposon insertion loci by iPBS marker 1882 resulted in seven different locus levels with the obtained low polymorphism at the level of 48 %. This marker provided the lowest amount of amplicons **(table 1)** per accessions with the maximum of 5 and minimum of 2 generated amplicons with the length varied from 200 up to the 750 base pairs. Dendrogram constructed from 1882 marker generated data has grouped the peanut accessions into two main clusters with a very different iPBS profile for accession no. 20 that was separated from the other accessions at the level of UPGMA dissimilarity of 0.55 (dendrogram not shown). The Jaccard coefficient of genetics dissimilarity has ranged from 0.00 up to the 0.71. When analyzed the peanut germplasm collection by iPBS marker 1882, eighteen from the accession was not possible to separate based on the obtained iPBS fingerprints. These were grouped into five alone standing sub clusters with the following group distribution of accessions – (1,13,14,15); (7,16); (2,3,5,6,8,11,12,17); (4,21) and (9,18) – all of these possess the Jaccard coefficient of genetic dissimilarity 0.00 **(figure 1)**.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	0.00																				
2	0.33	0.00																			
3	0.33	0.00	0.00																		
4	0.50	0.25	0.25	0.00																	
5	0.33	0.00	0.00	0.25	0.00																
6	0.33	0.00	0.00	0.25	0.00	0.00															
7	0.33	0.50	0.50	0.25	0.50	0.50	0.00														
8	0.33	0.00	0.00	0.25	0.00	0.00	0.50	0.00													
9	0.60	0.40	0.40	0.20	0.40	0.40	0.40	0.40	0.00												
10	0.50	0.25	0.25	0.40	0.25	0.25	0.60	0.25	0.50	0.00											
11	0.33	0.00	0.00	0.25	0.00	0.00	0.50	0.00	0.40	0.25	0.00										
12	0.33	0.00	0.00	0.25	0.00	0.00	0.50	0.00	0.40	0.25	0.00	0.00									
13	0.00	0.33	0.33	0.50	0.33	0.33	0.33	0.33	0.60	0.50	0.33	0.33	0.00								
14	0.00	0.33	0.33	0.50	0.33	0.33	0.33	0.33	0.60	0.50	0.33	0.33	0.00	0.00							
15	0.00	0.33	0.33	0.50	0.33	0.33	0.33	0.33	0.60	0.50	0.33	0.33	0.00	0.00	0.00						
16	0.33	0.50	0.50	0.25	0.50	0.50	0.00	0.50	0.40	0.60	0.50	0.50	0.33	0.33	0.33	0.00					
17	0.33	0.00	0.00	0.25	0.00	0.00	0.50	0.00	0.40	0.25	0.00	0.00	0.33	0.33	0.33	0.50	0.00				
18	0.60	0.40	0.40	0.20	0.40	0.40	0.40	0.40	0.00	0.50	0.40	0.40	0.60	0.60	0.60	0.40	0.40	0.00			
19	0.60	0.40	0.40	0.20	0.40	0.40	0.40	0.40	0.33	0.50	0.40	0.40	0.60	0.60	0.60	0.40	0.40	0.33	0.00		
20	0.60	0.66	0.66	0.50	0.66	0.66	0.40	0.66	0.33	0.71	0.66	0.66	0.60	0.60	0.60	0.40	0.66	0.33	0.33	0.00	
21	0.50	0.25	0.25	0.00	0.25	0.25	0.25	0.25	0.20	0.40	0.25	0.25	0.50	0.50	0.50	0.25	0.25	0.20	0.20	0.50	0.00

Figure 1 Jaccard coefficient values in the analyzed peanut germplasm accessions by iPBS marker 1882

Amplification of retrotransposon insertion loci by iPBS marker 2079 resulted in twelve different locus levels with the obtained polymorphism at the level of 67 %. This marker provided the amount of amplicons **(table 1)** per accessions with the maximum of 10 and minimum of 6 generated amplicons with the length varied from 100 up to the 800 base pairs. Dendrogram constructed from 2079 marker generated data has grouped the peanut accessions into three main clusters

(dendrogram not shown). In this case, no very different iPBS fingerprint profile was generated for none of the analyzed accessions. All the generated clusters were joined at the level of UPGMA dissimilarity of 0.4. The Jaccard coefficient of genetics dissimilarity has ranged from 0.00 up to the 0.54 **(figure 2)**. In the case of this marker, only eight from the accession was not possible to separate based on the obtained iPBS fingerprints. These were grouped into four alone standing sub clusters with the following group distribution of accessions – (1, 8); (4, 5); (6, 17) and (18, 19).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	0.00																				
2	0.14	0.00																			
3	0.33	0.22	0.00																		
4	0.14	0.25	0.40	0.00																	
5	0.14	0.25	0.40	0.00	0.00																
6	0.25	0.12	0.11	0.33	0.33	0.00															
7	0.33	0.22	0.36	0.40	0.40	0.30	0.00														
8	0.00	0.14	0.33	0.14	0.14	0.25	0.33	0.00													
9	0.25	0.33	0.45	0.33	0.33	0.40	0.11	0.25	0.00												
10	0.33	0.40	0.20	0.40	0.40	0.30	0.36	0.33	0.30	0.00											
11	0.33	0.22	0.20	0.40	0.40	0.30	0.20	0.33	0.30	0.20	0.00										
12	0.37	0.25	0.40	0.44	0.44	0.33	0.22	0.37	0.33	0.40	0.22	0.00									
13	0.54	0.45	0.41	0.45	0.45	0.36	0.27	0.54	0.36	0.41	0.41	0.30	0.00								
14	0.50	0.40	0.50	0.40	0.40	0.45	0.20	0.50	0.30	0.50	0.36	0.22	0.10	0.00							
15	0.44	0.33	0.45	0.33	0.33	0.40	0.30	0.44	0.40	0.45	0.30	0.12	0.20	0.11	0.00						
16	0.44	0.33	0.45	0.33	0.33	0.40	0.30	0.44	0.40	0.58	0.45	0.33	0.20	0.11	0.22	0.00					
17	0.25	0.12	0.11	0.33	0.33	0.00	0.30	0.25	0.40	0.30	0.30	0.33	0.36	0.45	0.40	0.40	0.00				
18	0.25	0.33	0.11	0.33	0.33	0.22	0.45	0.25	0.40	0.11	0.30	0.50	0.50	0.58	0.54	0.54	0.22	0.00			
19	0.25	0.33	0.11	0.33	0.33	0.22	0.45	0.25	0.40	0.11	0.30	0.50	0.50	0.58	0.54	0.54	0.22	0.00	0.0		
20	0.33	0.22	0.20	0.22	0.22	0.11	0.36	0.33	0.45	0.36	0.36	0.40	0.27	0.36	0.30	0.30	0.11	0.30	0.30	0.00	
21	0.25	0.12	0.11	0.33	0.33	0.22	0.30	0.25	0.40	0.30	0.11	0.33	0.50	0.45	0.40	0.40	0.22	0.22	0.22	0.30	0.00

Figure 2. Jaccard coefficient values in the analyzed peanut germplasm accessions by iPBS marker 2079

Amplification of retrotransposon insertion loci by iPBS marker 2274 resulted in thirteen different locus levels with the obtained polymorphism at the level of 67 %. This marker provided the amount of amplicons **(table 1)** per accessions with the maximum of 12 and minimum of 6 generated amplicons with the length varied from 100 up to the 800 base pairs **(figure 3)**. Dendrogram constructed from 2274 primer generated data has grouped the peanut accessions into three main clusters, but the level of UPGMA dissimilarity, that was lowest from all of the three used iPBS markers. In this case, two accessions (1, 12) generated very different iPBS fingerprint profile that separated them from all of the others at the level of 0.24. All the generated clusters were joined at the level of UPGMA dissimilarity of 0.4. The Jaccard coefficient of genetics dissimilarity has ranged from 0.00 up to the 0.5 **(figure 4)**. In the case of this marker, sixteen of analyzed accessions was not possible to separate based on the obtained iPBS fingerprints (dendrogram not shown). These were grouped into five alone standing sub clusters with the following group distribution of accessions – (1,12); (2,9,10,11,14,16); (13,17); (18,19) and (4,7,8,15).

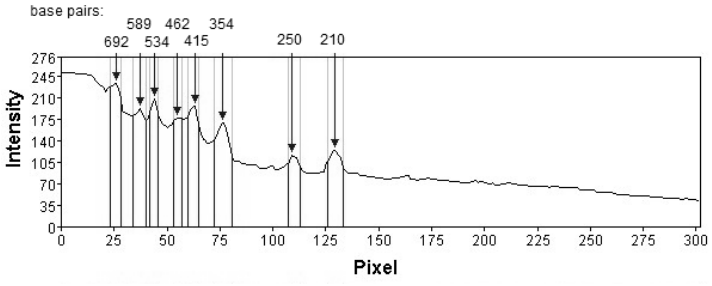

Figure 3 iPBS profile of accession 5 of peanut when iPBS marker 2274 used.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	0.00																				
2	0.45	0.00																			
3	0.33	0.18	0.00																		
4	0.33	0.18	0.20	0.00																	
5	0.40	0.25	0.27	0.10	0.00																
6	0.40	0.09	0.10	0.10	0.18	0.00															
7	0.33	0.18	0.20	0.00	0.10	0.10	0.00														
8	0.33	0.18	0.20	0.00	0.10	0.10	0.00	0.00													
9	0.45	0.00	0.18	0.18	0.25	0.09	0.18	0.18	0.00												
10	0.45	0.00	0.18	0.18	0.25	0.09	0.18	0.18	0.00	0.00											
11	0.45	0.00	0.18	0.18	0.25	0.09	0.18	0.18	0.00	0.00	0.00										
12	0.00	0.45	0.33	0.33	0.40	0.40	0.33	0.33	0.45	0.45	0.45	0.00									
13	0.40	0.09	0.10	0.27	0.33	0.18	0.27	0.27	0.09	0.09	0.09	0.40	0.00								
14	0.45	0.00	0.18	0.18	0.25	0.09	0.18	0.18	0.00	0.00	0.00	0.45	0.09	0.00							
15	0.33	0.18	0.20	0.00	0.10	0.10	0.00	0.00	0.18	0.18	0.18	0.33	0.27	0.18	0.00						
16	0.45	0.00	0.18	0.18	0.25	0.09	0.18	0.18	0.00	0.00	0.00	0.45	0.09	0.00	0.18	0.00					
17	0.40	0.09	0.10	0.27	0.33	0.18	0.27	0.27	0.09	0.09	0.09	0.40	0.00	0.09	0.27	0.09	0.00				
18	0.45	0.16	0.18	0.33	0.38	0.25	0.33	0.33	0.17	0.17	0.17	0.45	0.09	0.17	0.33	0.16	0.09	0.00			
19	0.45	0.16	0.18	0.33	0.38	0.25	0.33	0.33	0.17	0.17	0.17	0.45	0.09	0.17	0.33	0.17	0.09	0.00	0.00		
20	0.25	0.27	0.11	0.11	0.20	0.20	0.11	0.11	0.27	0.27	0.27	0.25	0.20	0.27	0.11	0.27	0.20	0.27	0.27	0.00	
21	0.50	0.08	0.25	0.25	0.31	0.17	0.25	0.25	0.08	0.08	0.08	0.50	0.17	0.08	0.25	0.08	0.17	0.08	0.08	0.33	0.00

Figure 4 Jaccard coefficient values in the analysed peanut germplasm accessions by iPBS marker 2274

For all of the primer-individual constructed dendrogram, a very high cophenetic coefficients were achieved **(table 1)** but, the low polymorphic information content values. We suppose, that this is a result of a quite conserved insertions of individual retrotransposon families in the genome of *Arachis hypogaea*, L. ***Nascimento et al (2018)*** has analysed the distribution of LTR segments of retrotransposons in the genome of peanut with the result of the dispersion mainly on arms and proximal regions of most of the chromosomes of *Arachis hypogaea*, L.. Up to now, sequences of retrotransposons Fidel ***(Nielen et al., 2010)***, Feral, Pipa and Pipoka were extracted from the BAC clones of peanut genome. All of them are presented as complete or in a manner of numerous isolated LTRs of these autonomous Ty3-*gypsy* type elements ***(Bertioli et al., 2013)***. Other Ty-3 gypsy elements that were described in the genome of Arachis hypogaea, L. are sequences of Curu, RE138, Grilo, and Mico ***(Nielen et al., 2010, 2011)***.

iPBS sequences that were used as markers, were reported previously to be a part of different plant species such as *Solanum tuberosum*, *Brassica rapa*, *Peonia anomala* or *Digitalis grandiflora* (***Kalendar et al., 2010***). A complete sequence identity of 1882 primer exist with *Linum usitatissimum* LTR retrotransposon FL7 ***(Smýkal et al., 2011)***.

In order to further evaluate the performance of the iPBS markers and assess the genetic diversities among the varieties, the parameters of polymorphic information content (PIC) was calculated. This value is used to estimate the discriminating ability of a primer and is based on measurements of the efficiency of polymorphic loci in revealing genetic diversity among the varieties ***(Guo et al., 2014)***. In this study, the value of PIC ranged from 0.33 to 0.46 **(table 1)**, indicating that the iPBS markers are useful for evaluation of genetic variation of *Arachis hypogaea* L., because PIC for dominant markers is a maximum of 0.5 ***(De Riek et al., 2001)***.

When comparing all of the primer-unique dendrograms generated for analysed peanut germplasm, five of the accession (1,4,8,9,11 and 18) where not distinguishable by any of the primers used in the study, but when all the obtained iPBS amplicons were combined together, all of them are clearly separated in the resulted dendrogram **(figure 5)**.

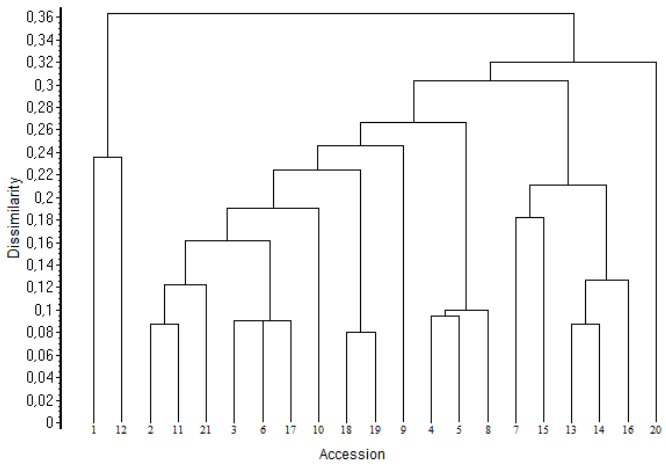

Figure 5 Dendrogram of analysed peanut accessions by combined iPBS generated fingerprints.

CONCLUSION

We have characterized the genomic iPBS fingerprint polymorphism in group of 21 peanut genotypes that were collected *in situ*. The analysis showed that the iPBS markers are an easy applicable and effective DNA based markers for the assesment of the genetic diversity in peanut natural germplasm. The dendrograms constructed on UPGMA algorithm divided 21 analyzed accessions into two main clusters in the case of individually evaluated primers used in the study and into a six balances clusters in the case of combining the results of individual primers. Using three iPBS primers, some of the peanuts accessions have not been differentiated for individual markers used, but the combined data differentiated all of the analysed accessions. This study consider iPBS markers as sufficiently polymorphic in the genome of peanut that will be useful in the assessment of its diversity and germplasm management for use in breeding and conservation.

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