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Research Article

Genetic relationships among *Arachis* species based on AFLP

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Abstract

Amplified Fragment Length Polymorphism (AFLP) was used to establish the genetic relationships among 20 species from seven of the nine sections of genus *Arachis*. The level of polymorphism among nine accessions of the cultivated peanut, *A. hypogaea* L., was also evaluated. Three combinations of primers were used to amplify the AFLPs. The fragments were separated in 6% denaturing acrylamide gels. A total of 408 fragments were analyzed. An average of 135.3 fragments per primer combination were scored, and the largest number of fragments was 169 using primer combination *Eco* RI - ACC / *Mse* I – CTG, while the lowest was 108, with *Eco* RI - ACT / *Mse* I - CTT. In general, the genetic relationships established using AFLPs agreed with the classification established using morphology and crossability data. The results indicated that AFLPs are good markers for establishing the relationships among *Arachis* species. The polymorphism detected in *A. hypogaea* by this method was higher than the one found with other markers, like RAPDs and RFLPs. However, our data suggest that the polymorphism detected be using AFLP with only three primer combinations is still too low to be used for any kind of genetic study in this species.

Key words: AFLP, *Arachis*, peanut, taxonomic relationship.

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Introduction

The genus *Arachis* was divided into nine sections by Krapovickas and Gregory (1994). Although those two authors analyzed a large number of accessions of several species, the large number of accessions recently collected broadened the geographic distribution of most sections. New questions about the taxonomic placement and relationships between some species have frequently been raised in this genus, since some accessions show morphological characteristics, which are intermediate between different species. Changes in the taxonomic relationships are likely to happen, when those new accessions are taken into account. If one considers the high probability of new materials being found, to be added to the *Arachis* germplasm collections, since a relevant effort has been made to broaden this collection, those changes might be quite significant. Thus, a methodology capable of helping to establish the genetic relationships within this genus in a reliable and fast way would be very useful.

Molecular markers have been used to establish taxonomic and phylogenetic relationships in several plant species (Fatokun *et al*., 1993; Xu and Slleper, 1994; Curtis *et al*., 1995; Cerry *et al*., 1996). The phylogenetic relationships in the genus *Arachis* have also been studied using different molecular markers, such as seed protein profiles (Singh *et al*., 1991; Singh *et al*., 1994), isoenzymes (Stalker *et al.,* 1994; Maass and Ocampo, 1995; Galgaro *et al*., 1997), RAPDs (Halward *et al*., 1991, 1992; Gimenes *et al.,* 2000), and RFLPs (Kochert *et al*., 1991; Paik-Ro *et al*., 1992; Stalker *et al*., 1995; Kochert *et al.,* 1996, Galgaro *et al*., 1998). All those markers have generated information that has been very useful for solving taxonomic and phylogenetic questions in this genus. However, they have some limitations, such as a low level of polymorphism, detection of few loci per assay, requirement of large quantities of relatively pure DNA, high cost, aside from being labor-intensive, time-consuming, offering very poor genetic information due the detection of just one allele per locus, and having a low reproducibility (Jain *et al.*, 1194; Millan *et al.*, 1996, Hill *et al.*, 1996; Paul *et al.*, 1997).

AFLP (Amplified Fragment Length Polymorphism), a PCR-based technique (Vos *et al.*, 1995), seems to overcome many of the limitations of the other molecular markers. AFLP has revealed much more polymorphism than RAPD and RFLP, because it allows a larger number of loci to be sampled in a single assay (10 to 50 polymorphic loci), it is more reliable and easier to score than RAPDs, and requires less time to assay a large number of loci. Our objective was to evaluate the usefulness of AFLPs for the establishment of taxonomic relationships among the species

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of genus *Arachis*, and to evaluate the polymorphism among cultivars of *A. hypogaea.*

Material and Methods

Plant material

Nine morphologically diverse accessions of *A. hypogaea*, cultivated in different countries, and 19 wild species of *Arachis* from seven sections of the genus were analyzed (Table I).

DNA extraction

DNA was extracted from leaves, using the procedure described by Kochert *et al.* (1991). The DNA quality was checked in 0.8% agarose gels, and its quantity measured by using a 260 nm wavelength. For the AFLP analysis, the DNAs were diluted to 14 ng/µL.

Table I - Accessions of *A. hypogaea* and other *Arachis* species analyzed.

Section	Species name	Accession
Arachis	*A. hypogaea*	
		PI 280688[1]
		PI 275690[2]
		PI 262090[3]
		PI 268906[4]
		PI 139914[5]
		PI 262118[6]
		PI 261924[7]
		cv. GK 3[8]
		PI 109839[9]
	A. monticola	K 30062
	A. ipaënsis	K 30076
	A. duranensis	G 10038
	A. glandulifera	K 30091
	A. diogoi	K 30001
	A. correntina	K 7830
	A. kuhlmannii	K 30017
	A. kempff-mercadoi	K 30088
	A. stenosperma	H 410
Caulorrhizae	*A. pintoi*	V 13364
	A. repens	PI 338277
Rhizomatosae	*A. burkartii*	H 17
	A. glabrata	V 11922
Erectoides	*A. paraguariensis*	V 7669
	A. major	V 7644
Extranervosae	*A. macedoi*	-
	A. burchellii	V12618
Procumbentes	*A. rigonii*	G 10034
Heteranthae	*A. dardani*	G 12943

Collector's and Institutional abbreviations: PI = Plant Introduction, G = Gregory, H = Hammons, K = Krapovickas, V = Valls. Origin of each accession of *A. hypogaea*: 1: Peru; 2: Brazil; 3: Bolivia; 4: Zimbabwe, 5: Zaire; 6: Bolivia; 7: Argentina; 8: USA; 9: Venezuela.

AFLP analysis

The AFLP analysis was performed following the manual of the AFLP kit (Life Technologies), with minor modifications. 250 ng (18 µL of the 14 ng/µL DNA dilution) of genomic DNA were digested for 2 hours at 37 °C, using 3 µL of a mix of *Eco* RI and *Mse* I. The restriction enzymes were inactivated by heating at 70 °C for 15 min. *Eco* RI and *Mse* I adapters were ligated to the restriction fragments, adding to each tube 24 µL of adapter ligation solution, 1 µL of T4 DNA ligase, and incubating the tubes at 20 °C for 2 h. A pre-amplification reaction was performed, mixing 5 µL of a 1:10 dilution of the digested and ligated fragments, 40 µL of pre-amp primer mix, 5 µL of 10 X PCR buffer, and 1 µL of 1 unit/µL *Taq* DNA polymerase, in a final volume of 51 µL. The following pre-amplification cycle was performed 20 times: 94 °C for 30 s, 56 °C for 60 s, and 72 °C for 60 s. The pre-amplification solution was diluted to 1:50 (v/v), and 3 µL were transferred to a 1.5 mL tube containing 147 µL of TE buffer. A selective amplification was performed, mixing 5 µL of a mix of 5 µL of labeled *Eco* RI primer (18 µL of *Eco* RI primer, 10 µL of kinase buffer, 20 µL of alpha ^{32}P, 2 µL of T4 polynucleotide Kinase) and 45 µL of *Mse* I primer, 5 µL of a mix of 79 µL of millipore water, 20 µL of 10 X PCR buffer and 1 µL of *Taq* DNA polymerase, and 5 µL of diluted pre-amplification solution. The selective amplifications were performed using the three pairs of primers (*Eco* RI - AGC / *Mse* I - CTG; *Eco* RI - ACT / *Mse* I - CTT; *Eco* RI - ACC / *Mse* I - CTG). A touch-down cycle was performed: 1 cycle of 60 s at 94 °C, 60 s at 65 °C and 90 s at 72 °C; followed by 9 cycles of 1 °C lower annealing temperature each cycle, and 23 cycles of 30 s at 94 °C, 30 s at 56 °C and 60 s at 72 °C. The pre and the selective amplifications were performed in a Perkin-Elmer Thermocycler.

Gel electrophoresis

Twenty microliters of loading buffer (98% formamide, 10 mM EDTA, 0.005% of xylene cyanol FF and 0.005% of bromophenol blue) were added to each tube. The PCR products were denatured by heating at 90 °C for 3 min, and then immediately placed on ice. Two microliters of each sample were loaded per track, and the fragments were electrophoresed in 6% acrylamide gels (20:1 acrylamide:bis; 7.5 M urea; 1 X TBE) for 1.30 h at 55 W. The gel was transferred to a 3MM chromatography paper and dried in a gel drier at 80 °C for 2 h. Gels were exposed to XOMAT X-ray film (KODAK) overnight at room temperature.

Data analysis

The AFLP bands were scored for presence or absence in each accession. Fragments of the same size were considered to have the same sequence. A genetic distance matrix was obtained using the genetic distance described by Link *et al.* (1995), and used to construct a UPGMA tree. A bootstrap analysis was performed using 1,000 replicates. All analyses were performed using the TREECON computer (program) software (Van de Peer and Watcher, 1994).

Results

A total of 408 fragments were analyzed using the three pairs of primers. One hundred and eight fragments were scored in the essay performed by using the pair of primers *Eco* RI-ACT / *Mse* I-CTT, 131 with *Eco* RI-AGC / *Mse* I-CTG, and 169 with *Eco* RI-ACC / *Mse* I-CTG, with an average of 136 fragments per pair of primers used. Four hundred and six fragments were polymorphic, with an average of 135.3 per reaction. Only two fragments were monomorphic in all samples analyzed.

A total of 94 fragments were detected in the accessions of *A. hypogaea*, and only six of those fragments were polymorphic (6.4%). Figure 1 shows a polymorphic fragment detected in *A. hypogaea* with primer combination *Eco* RI-ACC / *Mse* I-CTG.

The relationships among the species analyzed are shown in Figure 2. The species were grouped into three major clusters. The first cluster comprised all the species analyzed of section *Arachis,* the second one comprised the species analyzed of sections *Caulorrhizae* (*A. pintoi* and *A. repens*), *Rhizomatosae* (*A. burkartii* and *A. glabrata*), *Heteranthae* (*A. dardanii*), Procumbentes (*A. rigonii*) and *Erectoides* (*A. paraguariensis*, *A. major*); the third group comprised *A macedoi* and *A. burchellii*, both of section *Extranervosae*. The group formed by the species of section *Arachis* was subdivided into three subgroups: the first one comprised all the accessions of *A. hypogaea, A. monticola*, *A ipaënsis* and *A. duranensis*, the second one comprised *A. kempff-mercadoi, A. diogoi*, *A. kuhlmannii*, *A. stenosperma*, *A. correntina*, and the third one comprised *A. glandulifera*. The bootstrap values ranged from 24% to 100%.

The two analyzed species of section *Rhizomatosae* did not group together. *Arachis burkartii* was grouped closer to *A. pintoi* and *A. repens*, while *A. glabrata* was placed closer to *A. paraguariensis* and *A. major,* both of section *Erectoides*.

Discussion

Four hundred and eight fragments were detected using three pairs of primers, 406 (99%) being polymorphic, taking all the species into account. The RAPD analysis of five species of section *Arachis* using six primers showed an average of 19.3 polymorphic loci (Lanham *et al.*, 1992).

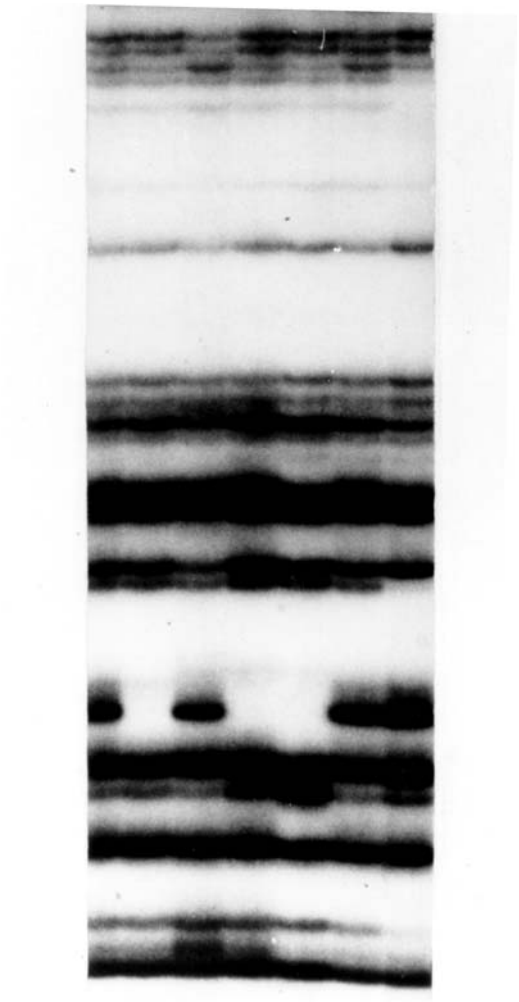

Figure 1 - Polymorphism among the accessions of *A. hypogaea* using the primers *Eco* RI - ACC/ *Mse* I - CTG.

Lanham *et al.* (1994) analyzed seed storage protein variation in seventy-two accessions of 22 species of sections *Arachis*, *Erectoides*, *Extranervosae* and *Triseminatae* and found a total of 24 polymorphic bands.

The results suggest that AFLP is very useful in establishing the genetic relationships among *Arachis* species. As a rule, the grouping of the species based on AFLP data agreed with the classification established by using morphology, geographic distribution and cross-compatibility (Krapovickas and Gregory, 1994), despite the small number of accessions analyzed of each species. AFLP data were also consistent with the existing knowledge of the systematics of other plant species (Hill *et al.*, 1996; Paul *et al.*, 1997).

The species of section *Arachis* were grouped into three clusters. The first one comprised *A. hypogaea* (AABB), *A. monticola* (AABB), *A. duranensis* (AA) and *A. ipaënsis* (BB). The close relationship of the diploid species *A. ipaënsis* and A. *duranensis* with *A. hypogaea* is consistent with previous studies (Kochert *et al.*, 1991; Kochert *et*

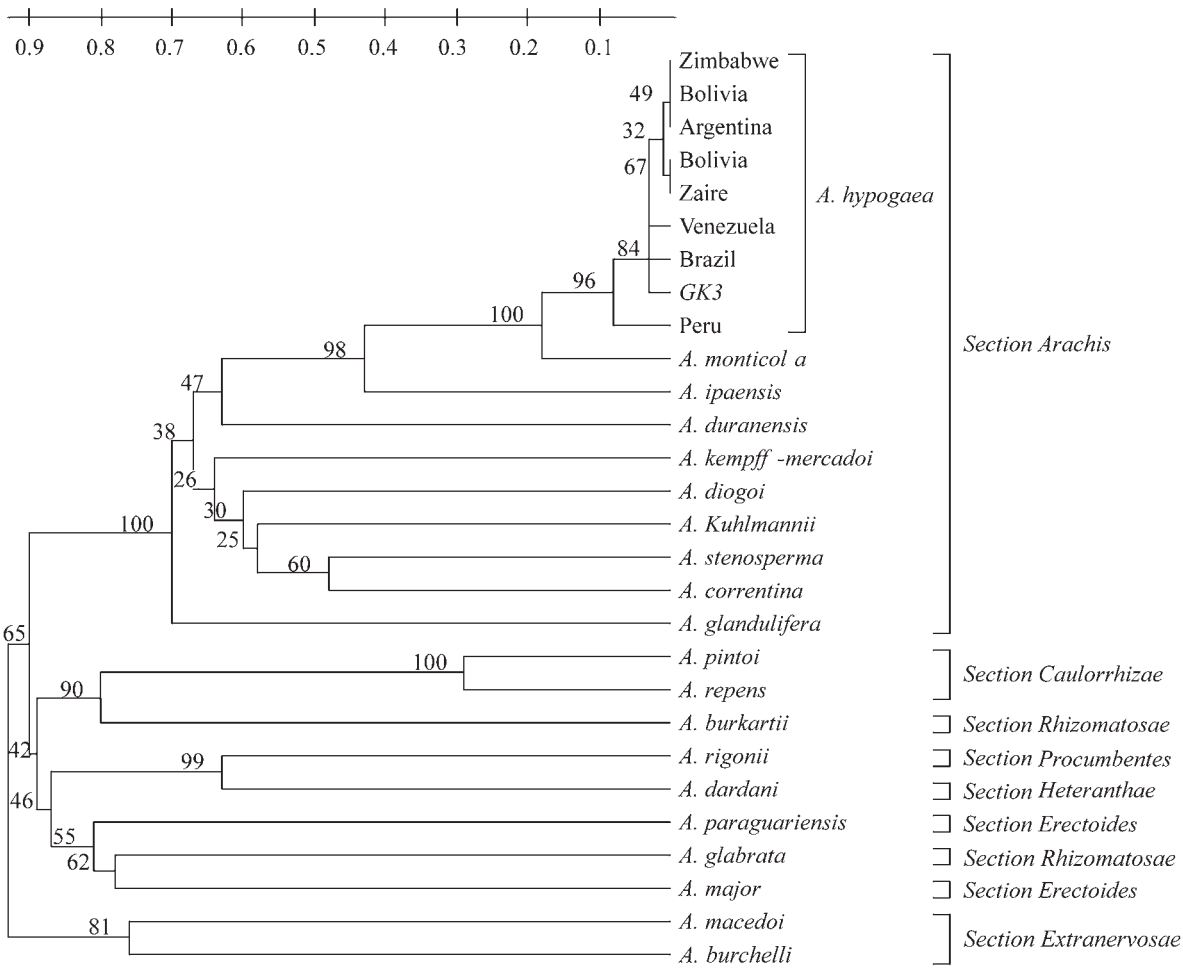

Figure 2 - UPGMA tree showing the relationships among the 20 species of seven sections of the genus *Arachis*.

al., 1996), which also appointed these diploid species as being the most probable donors of the genomes of *A. hypogaea*. The second group comprised the A genome species, and the third one comprised *A. glandulifera*, which has a D genome (Stalker, 1991). The data agreed with the cytogenetic classification, that is a good evidence of crossability among A genomes species.

The other sections with more than one species analyzed were also well characterized. *Arachis pintoi* and *A. repens* (section *Caulorrhizae*) were grouped very close to each other. Hybrids between *A. pintoi* and *A. repens* show a pollen fertility level of 86.9% (Gregory and Gregory, 1979). RAPD analysis of several accessions of *A. pintoi* and *A. repens* indicated that these two species are closely related on molecular grounds (Gimenes *et al.*, 2000). The species of section *Erectoides* (*A. paraguariensis*, *A. major*) were grouped with *A. glabrata*, that belongs to section *Rhizomatosae*. Section *Extranervosae* was also characterized, having *A. macedoi* and *A. burchellii* grouped together. The exception was section *Rhizomatosae*, which had *A. glabrata* and *A. burkartii* put apart in the tree. *Arachis glabrata* is tetraploid, while *A. burkartii* is diploid, and it is known that the latter one does not cross with any other species of genus *Arachis* (Gregory and Gregory, 1979). Thus, the evidence suggests that these two species are not closely related and, along the evolution and diversification of this genus, rhizomes may have appeared independently, at least twice.

Six fragments were polymorphic in *A. hypogaea* cultivars. The polymorphism detected using this molecular marker is still very low, when compared to other crops. For instance, in an analysis of 14 rice accessions, 27.8% (147/529) of loci detected were polymorphic (Mackill *et al.*, 1996). The percentage of polymorphic markers found in *A. hypogaea* (6.4%) was similar to the one found in another AFLP analysis of variation within 6 divergent genotypes from three botanical varieties of *A. hypogaea* (He and Prakash, 1997). Because *A. hypogaea* may have arisen recently from a single polyploidization event (Halward *et al.*, 1991), it is likely that a simple nucleotide substitution, rather than gross differences in few loci, may account for the variation in the cultivated peanut (Kochert *et al.*, 1996; He and Prakash, 1997). The microsatellite loci analysis of *A. hypogaea*, where alleles differed from each other in a

few bases, showed higher polymorphism than RFLP and RAPDs (Hopkins *et al.*, 1999). Thus, the data presented in this and in other studies indicate that techniques which allow the detection of few to single nucleotide modifications, such as SNPs (Single Nucleotide Polymorphisms), will have to be used in *A. hypogaea* to identify informative loci.

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