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# RFLP variability in peanut (*Arachis hypogaea* L.) cultivars and wild species

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**Summary**. RFLP variability was studied in eight U.S. peanut cultivars, representing the four market types, and in 14 wild *Arachis* species accessions, using random genomic clones from a PstI library. Very low levels of RFLP variability were found among the allotetraploids, which included the U.S. cultivars and *Arachis monticola*, a wild species. The diploid wild species were very diverse, however. RFLP patterns of the allotetraploids were more complex than the diploids, and the two constituent genomes could usually be distinguished. On the basis of RFLP band sharing, *A. ipaensis*, *A. duranensis*, and *A. spegazzinii* appeared most closely related to the diploid progenitor species of the allotetraploids. A dendrogram of relationships among the diploid wild species was constructed based on band sharing.

**Key words**: Peanut – RFLP – Plant breeding – Genetic diversity

## Introduction

The cultivated peanut (*Arachis hypogaea* L.) is an allotetraploid (2n = 4x = 40) of South American origin. It has been cultivated since prehistoric times, and peanut remains have been found in the tombs of Native Americans dating back to 500–750 B.C. (Hammons 1982). After the European conquest of the New World, peanuts were transported to Europe, Africa, Asia, and later to North America. Today the peanut is recognized as one of the most important international crops.

The genus *Arachis* has been estimated to consist of about 70 species, classified into seven sections on the basis of morphological characteristics and cross compatibility (Gregory et al. 1973). Most species are diploids (2n = 2x = 20), but tetraploids have arisen independently in two sections (Smartt and Stalker 1982). The section Arachis consists of 14 named species (as well as a number of unnamed accessions), including two tetraploid forms: *A. hypogaea*, the cultivated peanut, and *A. monticola*, a wild tetraploid form that is completely cross-fertile with cultivated peanut and is the assumed direct progenitor of cultivated peanut (Stalker and Moss 1987). The diploid species in section *Arachis* generally can be hybridized. Most interspecific crosses produce semifertile $F_1$ hybrids, with the exception of those crosses in which *A. batizocoi* or *A. spinaclava* is one of the parents. Diploid species in section *Arachis* are cross-compatible with cultivated peanut to varying degrees and typically produce sterile triploid hybrids. The tetraploid members behave as allotetraploids and appear to be highly diploidized, with regular bivalent formation at meiosis and disomic inheritance (Smartt and Stalker 1982; Wynne and Coffelt 1982). The taxonomic relationships within the group have not been conclusively delineated, and it is not clear which diploid species are the likely progenitors of the tetraploids, although some evidence is available from seed protein profiles and cytogenetic analysis (Smartt et al. 1978; Singh and Moss 1982, 1984; Klozová et al. 1983; Krishna and Mitra 1988; Singh 1988).

Cultivated peanuts are normally self-pollinating, and only a low degree of outcrossing apparently occurs (Wynne and Coffelt 1982). Little is known about the mating systems of the wild species, but they are also assumed to be self-pollinating. The geocarpic habit of peanuts is an unusual feature of their life cycle that has interesting consequences for dispersal and population structure. Seeds appear to be dispersed largely by water, and species distribution coincides to a large extent with major river systems. A great deal is known about certain aspects of peanut cultivation and production, and peanut

breeders have produced improved cultivars with high yield and varying levels of resistance to a number of insects and pathogens. However, few reports are available concerning genetic variability in peanuts as measured by isozymes or molecular markers, linkage groups, or genetic maps. To date, no genes have been localized on any given peanut chromosome. Our objectives were to investigate restriction fragment length polymorphisms (RFLPs) of nuclear DNA in cultivated peanut and some of its wild diploid relatives in an effort to learn more about levels of molecular genetic variability in peanut, to investigate systematic relationships among cultivated and wild forms, and to estimate the feasibility of constructing an RFLP map.

## Materials and methods

### *Germplasm*

To study genetic variability in U.S. peanut cultivars, two representatives were selected from each of the four market types (Virginia, runner, ssp. *hypogaea* var. *hypogaea*; Spanish, ssp. *fastigiata* var. *vulgaris*; Valencia, ssp. *fastigiata* var. *fastigiata*) commonly grown in the U.S. Other species in the section *Arachis* were represented by *A. monticola, Arachis* × *A. batizogaea*, nine named wild species, and four unnamed wild species accessions from section *Arachis* (Table 1). All plants were grown from seed and maintained in the greenhouse.

### *DNA isolation*

DNA was extracted from single plants of each accession by a modification of the Murray and Thompson (1980) procedure as described by Bernatzky and Tanksley (1986). Ten to twenty grams of leaf material was suspended in 150 ml of cold extraction buffer, ground in a Waring blender (15 s, medium speed), and centrifuged at $2{,}000 \times g$ for 15 min. The resulting crude nuclear preparation was suspended in nuclei lysis buffer, heated at 65 °C for 15 min, and extracted with chloroform-isoamyl alcohol. After precipitation with ethanol, the DNA was dissolved in TE (10 m*M* TRIS-CL, pH 7.0, 1 m*M* NaEDTA). DNA quantity was estimated by $OD_{260}$, and quality was determined by electrophoresis in agarose gels.

### *Library construction*

Total DNA from the peanut cultivar GK-7 was digested with PstI and subjected to electrophoresis in a 0.8% low melting point agarose gel. The region containing approximately 1–2 kb fragments was excised from the gel, and the DNA was extracted by melting the gel at 65 °C, followed by phenol extraction and precipitation. The isolated peanut DNA fragments were then ligated into phosphatased pUC8 plasmid vectors and transformed into *E. coli* DH5a cells by standard procedures.

### *Library characterization*

Recombinant clones were selected on IPTG-X-gal plates (Sambrook et al. 1989) and plasmids were isolated by a miniprep procedure (Wilimzig 1985). Recombinant plasmids were digested with PstI and subjected to electrophoresis on 0.8% agarose gels. The molecular weight of each insert was determined by comparison to molecular weight standards and blots were prepared on nylon filters by the method of Southern (1975). Total peanut DNA (GK-7) was labelled with $^{32}$P-dCTP by Nick-translation (Rigby et al. 1977) and hybridized to the filters to detect inserts containing repeated sequences. Cotton chloroplast DNA (a gift from G. Galau) was used to detect which members of the library contained chloroplast DNA inserts. Those inserts that showed no signal with either chloroplast DNA or total peanut DNA were assumed to represent low-copy-number nuclear sequences and were selected for RFLP analysis.

**Table 1.** Cultivars and wild species evaluated for RFLP variation

| Cultivar/wild species | Market type/ accession no. | Ploidy level |
|---|---|---|
| Toalson | Spanish | 2n = 4x = 40 |
| Pronto | Spanish | 2n = 4x = 40 |
| New Mexico Valencia A | Valencia | 2n = 4x = 40 |
| Valencia McRan | Valencia | 2n = 4x = 40 |
| Florigiant | Virginia | 2n = 4x = 40 |
| GK-3 | Virginia | 2n = 4x = 40 |
| Florunner | Runner | 2n = 4x = 40 |
| Okrun | Runner | 2n = 4x = 40 |
| *A. monticola* Krap. et Rig. | 30062 | 2n = 4x = 40 |
| *A. batizocoi* Krap. et Greg. | 9484 | 2n = 2x = 20 |
| *A. cardenasii* Krap. et Greg. *nom. nud.* | 10017 | 2n = 2x = 20 |
| *A. chacoense* Krap. et Greg. *nom. nud.* | 10602 | 2n = 2x = 20 |
| *A. duranensis* Krap. et Greg. *nom. nud.* | 30064 | 2n = 2x = 20 |
| *A. ipaensis* Greg. et Greg. *nom. nud.* | 30076 | 2n = 2x = 20 |
| *Arachis* species | 9660 | 2n = 2x = 20 |
| *Arachis* species | 30011 | 2n = 2x = 20 |
| *Arachis* species | 30017 | 2n = 2x = 20 |
| *Arachis* species | 35005 | 2n = 2x = 20 |
| *A. spegazzinii* Greg. et Greg. *nom. nud.* | 10038 | 2n = 2x = 20 |
| *A. spinaclava* | 30098 | 2n = 2x = 20 |
| *A. stenosperma* Greg. et Greg. *nom. nud.* | 410 | 2n = 2x = 20 |
| *A. villosa* Benth. | 3112 | 2n = 2x = 20 |
| *Arachis* × *batizogaea* | | 2n = 4x = 40 |

### *RFLP analysis*

Total DNA from the various peanut accessions was routinely digested overnight with the desired restriction enzyme and then subjected to electrophoresis on 1.2% agarose gels. Ten micrograms of peanut DNA was used in each gel lane. After electrophoresis, the DNA was transferred to nylon membranes (Southern 1975). DNA hybridization was performed using isolated inserts from the peanut genomic library, which were labelled with $^{32}$P-dCTP by the random primer technique (Feinberg and Vogelstein 1984). Autoradiograms were produced on Kodak X-Omat film with enhancing screens. Bands detected on the autoradiograms were scored and evaluated for the presence of RFLPs.

Parsimony analysis of RFLP data was carried out using the PAUP computer program developed by Dr. D.L. Swofford, Illinois Natural History Survey, Champaign/IL. Gel bands were organized into a 1-0 matrix and analyzed with the "branch and bound" method, using addition = stepwise and mulpar functions.

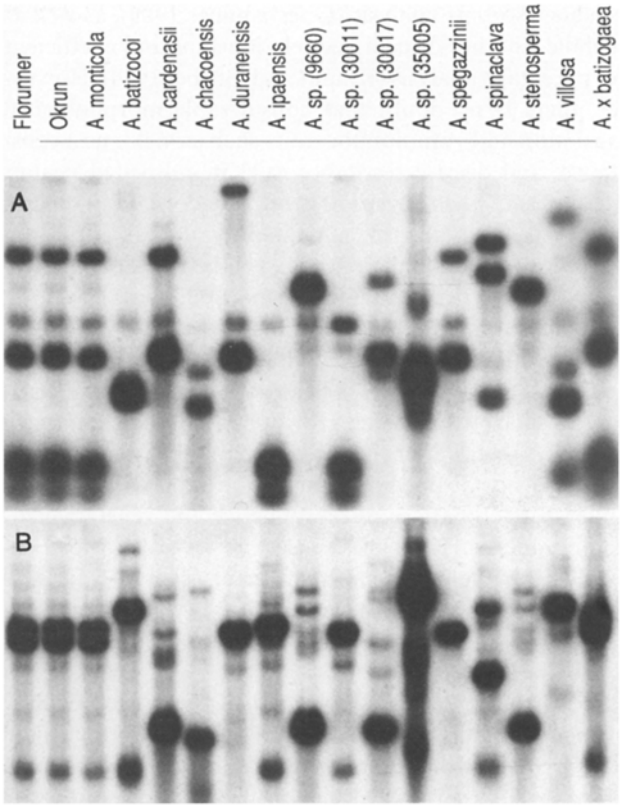


**Fig. 1 A and B.** Autoradiogramm of DNA from peanut cultivars and wild *Arachis* species, which had been digested with *Eco*RI and probed with two different random genomic clones from a *Pst*I library. **A** Probe GK78: *A cardenasii* or *A. spegazzini* could be combined with *A. ipaensis* or *Arachis* sp. (30011) to give the pattern found in the tetraploids. **B** Probe GK 77: *A. ipaensis* could be combined with *A. duranensis, Arachis* sp. (30011), or *A. spegazzinii* to give the tetraploid pattern

## Results

To select an enzyme to use for library construction, several six-cutter restriction enzymes were used to digest total peanut DNA. Several of these enzymes appeared to cut peanut DNA frequently, resulting in restriction fragments with a large range of sizes. However, PstI and SalI appeared to cut infrequently, and most of the DNA remained as high-molecular-weight fragments near the origin of the gel (data not shown). Selection of lower-molecular-weight fragments from digestion with methylation-sensitive enzymes such as PstI has been previously shown to enrich for low-copy-number sequences (Tanksley et al. 1987; Burr et al. 1988); therefore, PstI was selected for library construction. This method of selection appears to have been effective, since only about 10% of the clones in our library represented repeated sequences, based on our screening with labelled, total peanut DNA.

The peanut germplasm surveyed consisted of eight cultivars representing two each of the four major U.S. market types; *A. monticola*, a tetraploid wild species; *A.* × *batizogaea*, an interspecific hybrid between *A. hypogaea* and *A. batizocoi* (2n = 4 = 40) (Krapovickas et al. 1974); and accesions representing nine named and four unnamed diploid wild species within section *Arachis*. A total of 21 randomly chosen inserts from the genomic library and eight restriction enzyme digests (DraI, EcoRI, HaeIII, HindIII, HpaII, MspI, RsaI, ScaI) was used to probe this array of germplasm. Seven probes were useful for detecting polymorphism, revealing a variety of RFLP patterns, and most analyses were done with these seven probes. The most common result we obtained was little or no polymorphism among the cultivated peanuts and a great deal of RFLP variation among the diploid wild species. *A. monticola* and *A.* × *batizogaea* always produced RFLP patterns that were identical with those of the tetraploid cultivars (Fig. 1).

**Table 2.** Number of RFLP bands shared by *Arachis monticola* and various diploid wild *Arachis* species

| Species | Accession no. | Bands shared |
|---|---|---|
| *A. batizocoi* | 9484 | 13/31 |
| *A. cardenasii* | 10017 | 12/31 |
| *A. chacoensis* | 10602 | 9/23 |
| *A. duranensis* | 30064 | 22/31 |
| *A. ipaensis* | 30076 | 23/29 |
| *Arachis* species | 9660 | 14/31 |
| *Arachis* species | 30011 | 17/30 |
| *Arachis* species | 30017 | 9/30 |
| *Arachis* species | 35005 | 7/13 |
| *A. spegazzinii* | 10038 | 24/31 |
| *A. spinaclava* | 30098 | 11/30 |
| *A. stenosperma* | 410 | 12/31 |
| *A. villosa* | 3112 | 16/30 |

RFLP patterns of the tetraploid species were usually more complex than the diploids, and the two genomes present could often be distinguished by comparison with the diploid RFLP patterns. In an effort to identify potential diploid progenitors, we analyzed band sharing between tetraploids and diploids. Each diploid species had some bands in common with the tetraploids and some bands that were different, when analyzed over all probe/enzyme combinations. However, accessions of three diploid species, *A. ipaensis, A. duranensis*, and *A. spegazzinii*, were identified which more consistently shared bands with *A. monticola* (Table 2). When we attempted to reconstruct the tetraploid RFLP banding pattern by various combinations of the patterns derived from diploid species, *A. ipaensis*, combined with either *A. duranensis* or *A. spegazzinii*, was the combination that could most often reconstitute the tetraploid (Fig. 2).

Based on shared RFLPs, a dendrogram of the relationships among the peanut accessions was constructed using parsimony analysis (Fig. 3). Three groups were distinguished among diploid wild species. *Arachis duranen-*

| | A. cardenasii | A. chacoensis | A. duranensis | A. ipaensis | A. sp. (9660) | A. sp. (30011) | A. sp. (30017) | A. sp. (35005) | A. spegazzinii | A. spinaclava | A. stenosperma | A. villosa |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A. batizocoi | 3 | 0 | 5 | 2 | 0 | 1 | 0 | 0 | 5 | 1 | 0 | 1 |
| A. cardenasii | - | 0 | 1 | 5 | 2 | 1 | 2 | 0 | 2 | 1 | 1 | 1 |
| A. chacoensis | - | - | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| A. duranensis | - | - | - | 12 | 1 | 4 | 1 | 0 | 1 | 4 | 1 | 4 |
| A. ipaensis | - | - | - | - | 3 | 2 | 2 | 2 | 14 | 1 | 3 | 5 |
| A. sp. (9660) | - | - | - | - | - | 1 | 0 | 0 | 1 | 0 | 0 | 2 |
| A. sp. (30011) | - | - | - | - | - | - | 1 | 1 | 6 | 0 | 0 | 1 |
| A. sp. (30017) | - | - | - | - | - | - | - | 0 | 0 | 0 | 0 | 1 |
| A. sp. (35005) | - | - | - | - | - | - | - | - | 1 | 0 | 0 | 1 |
| A. spegazzinii | - | - | - | - | - | - | - | - | - | 3 | 1 | 3 |
| A. spinaclava | - | - | - | - | - | - | - | - | - | - | 0 | 0 |
| A. stenosperma | - | - | - | - | - | - | - | - | - | - | - | 2 |

**Fig. 2.** Pairs of diploid *Arachis* species that could be reconstituted to form the tetraploid pattern. Cell numbers represent the number of times a given pair of diploid species reconstituted the tetraploid pattern over all probe/enzyme combinations tested

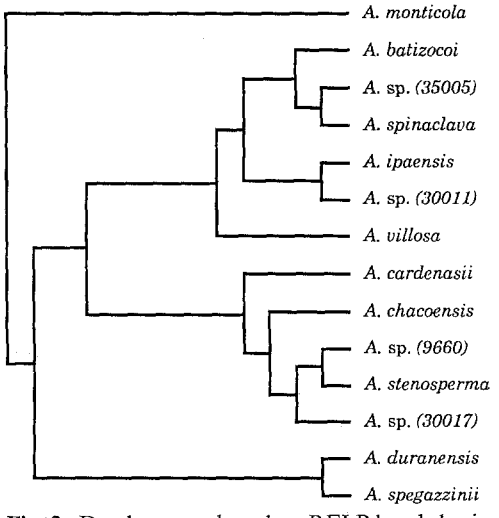


**Fig. 3.** Dendrogram, based on RFLP band sharing, of relationships among species of section Arachis

*sis* and *A. spegazzinii*, which we identified as potential contributors of one of the genomes of tetraploid peanut, appeared to be closely related. *Arachis ipaensis*, a likely contributor of the other genome, was in a separate group. On the basis of cytogenetics and crossing behavior, diploid wild species in section *Arachis* have been separated into "A" and "B" genomes. *Arachis batizocoi* is the sole "B" genome representative among the accessions we examined, but it did not form a separate group based on RFLP analysis.

## Discussion

Our results indicated a very low level of genetic diversity among U.S. peanut cultivars. This is in general agreement with findings using other experimental methods such as isozyme analysis (Grieshammer 1989), as well as detailed studies of pedigree relationships and coefficient of parentage analysis (Knauft and Gorbet 1989). However, there is no doubt that considerable morphological variation and variation for traits such as yield, pest resistance, and disease resistance exist in cultivated peanut germplasm. Such variation is often uncovered in crosses between subspecies of cultivated peanut. It is not yet clear how this seeming paradox will be resolved, but it is important to realize that major morphological and physiological variation may sometimes be caused by changes in only a few loci. For example, separation of cultivated peanuts into subspecies is based on morphological characters such as the overall growth habit of the plant (erect versus prostrate or bunch type versus runner type), whether or not flowers occur on the main stem, and the interspersion pattern of reproductive and vegetative lateral branches. These differences are reported to be controlled by one or a small number of genes (Wynne and Coffelt 1982) and would not necessarily be reflective of high general genetic diversity as measured by RFLPs or isozymes.

We found that *Arachis monticola* (accession 30062), which is usually classified as the only known tetraploid wild species in the section *Arachis*, is virtually identical in RFLP pattern to *A. hypogaea*. This is not completely unexpected, because the two species are known to be interfertile, with no apparent sterility of $F_1$ hybrids (Kirti et al. 1982) and a high degree of similarity of seed protein electrophoretic patterns (Klozová et al. 1983). The overall morphology of the two taxa is also similar. Previous studies of the chromosome pairing behavior of *A. hypogaea* and *A. monticola* have suggested that separating the two taxa into separate species may not be warranted (Kirti et al. 1982; Stalker and Moss 1987). Our results would support this conclusion.

The RFLP patterns of *A.* × *batizogaea* were also very similar to those of cultivated tetraploids. *A.* × *batizogaea* was developed from a cross between *A. batizocoi* and *A. hypogaea*, followed by four generations of selfing of the $F_1$ hybrid, during which time the chromosome number dropped to 40. Based on flavonoid patterns, it was believed that some introgression had taken place (Krapovickas et al. 1974). RFLP patterns were identical in this hybrid to the cultivars evaluated and suggest that *A. batizocoi* chromosomes may have been eliminated during the four generations of selfing and that, if introgression occurred, it must have been by recombination. A more comprehensive study using a large number of probe/enzyme combinations would be needed to detect any small segments of *A. batizocoi* chromosomes introgressed into the *A. hypogaea* genome.

The question of the diploid progenitors of tetraploid cultivated peanut has been the subject of several studies,

but no definitive conclusions have been reached. Smartt et al. (1978), on the basis of chromosome morphology, suggested that *A. cardenasii* and *A. batizocoi* might be progenitors and proposed that amphidiploids be constructed to test their hypothesis. Singh (1986, 1988) made extensive crosses of the diploid species in section Arachis, and studied chromosome behavior in the hybrids, constructed synthetic amphidiploids, and crossed these to cultivated peanut. Based on his observations, the most likely progenitors appeared to be *A. batizocoi* and *A. duranensis*. Our results are more complex, and it was not possible to identify unambiguously two species as the definite progenitors. Some probe/enzyme combinations clearly implicate certain diploid species. However, these same species seem to be ruled out when other probes are considered. When all probe/enzyme combinations are considered, band sharing analysis suggests that, of the accessions evaluated, *A. ipaensis* is likely to be one diploid progenitor. Either *A. duranensis* or *A. spegazzinii*, which have very similar RFLP patterns, could be the other progenitor. Based on the dendrogram generated using parsimony analysis (Fig. 3), *A. ipaensis* appears to belong to a different phylogenetic group than *A. spegazzinii* and *A. duranensis*, which clustered together as closely related branches on the dendrogram. Interestingly, *A. batizocoi*, which is almost always listed as one of the progenitors, does not receive much support from our RFLP analysis.

We believe that our work demonstrates that RFLP analysis can provide useful data for the analysis of phylogenetic and taxonomic questions in peanuts. However, more work is clearly needed. We need to examine more individuals of each accession to determine how much variability is present in each accession, more accessions to determine how much a species varies over its range, and other species from both the section *Arachis* and the other sections. As this data is accumulated, we should be able to determine how our results, based on a limited sampling, need to be modified.

Use of RFLP data in phylogenetic studies is fraught with difficulties. Bands of the same apparent molecular weight may not be allelic. Also, it is usually not known whether the observed RFLP variation is primarily the result of base changes or is caused by rearrangements such as insertions and deletions. If most RFLPs are caused by rearrangements, it becomes difficult to relate RFLP differences to nucleotide sequence differences. In spite of these uncertainties, the simple expedient of considering shared restriction fragments to be shared derived characters has worked well in some cases (Song et al. 1988, 1990). Therefore, our data was analyzed on this basis. The three groups we identified in the diploid wild species have no precedent in the literature, and the validity of this grouping will have to await further study involving additional accessions, additional plants from the same accessions, and additional probe/enzyme combinations.

Based on our results, there is not sufficient RFLP diversity to construct an RFLP map based on conventional technology and using a mapping population derived from a cross between tetraploid cultivars. Such a map will have to be constructed using an interspecific cross. Similar results have been found in tomato (Bernatzky and Tanksley 1986) and soybean (S. Tingey, personal communication), and RFLP maps were constructed with interspecific crosses. We are now constructing an RFLP map in peanut using an interspecific cross, and it should be of immediate use for monitoring introgression from wild species into cultivated peanut.

Our results have significance in terms of peanut breeding, which has largely concentrated on crosses among relatively narrow-based germplasm sources. In view of the apparent limited genetic variability available in such forms, it would seem that continued gains in peanut breeding would be hard to achieve and that new sources of pest resistance are not likely to be found in the elite germplasm pool utilized in the majority of peanut breeding programs. Using RFLPs to determine genetic relatedness among genotypes would allow peanut breeders to choose parental germplasm material to maximize variability in their breeding programs. The situation in peanut appears to be similar to that in tomato, where a low level of polymorphism exists in cultivars, and most current breeding programs in tomato concentrate on introgression from related wild species. We believe that peanut breeders may want to direct additional effort toward introgression from wild species. In the past, this has proven difficult because of ploidy differences, which necessitate the use of complex chromosomal manipulations. As molecular markers are developed, these will aid in both the identification of specific desirable genes in wild species of *Arachis* and in tracing their transmission once they are introgressed into cultivated germ plasm.

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