Transmission Genetics of Chromatin From a Synthetic Amphidiploid to Cultivated Peanut (*Arachis hypogaea* L.): Broadening the Gene Pool of a Monophyletic Polyploid Species

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Manuscript received September 20, 2000
Accepted for publication July 31, 2001

ABSTRACT

Polyploidy creates severe genetic bottlenecks, contributing to the genetic vulnerability of leading crops. Cultivated peanut is thought to be of monophyletic origin, harboring relatively little genetic diversity. To introduce variability from diploid wild species into tetraploid cultivated *Arachis hypogaea*, a synthetic amphidiploid {[*A. batizocoi* K9484 × (*A. cardenasii* GKP10017 × *A. diogoi* GKP10602)]$^{4\times}$} was used as donor parent to generate a backcross population of 78 progeny. Three hundred seventy RFLP loci were mapped onto 23 linkage groups, spanning 2210 cM. Chromatin derived from the two A-genome diploid ancestors (*A. cardenasii* and *A. diogoi*) comprised mosaic chromosomes, reflecting crossing over in the diploid A-genome interspecific F_1 hybrid. Recombination between chromosomes in the tetraploid progeny was similar to chromosome pairing reported for *A. hypogaea*, with recombination generally between chromosomes of the same subgenomic affinity. Segregation distortion was observed for 25% of the markers, distributed over 20 linkage groups. Unexpectedly, 68% of the markers deviating from expected segregation showed an excess of the synthetic parent allele. Genetic consequences, relationship to species origins, and significance for comparative genetics are discussed.

POLYPLOIDS comprise an estimated 30–80% of all angiosperm species, including many of the world's most important food, fiber, and forage crops (Stebbins 1971; Sanford 1983; Masterson 1994; Soltis and Soltis 1995). Although polyploid formation imposes a genetic bottleneck, the frequency of polyploidy in nature and the utility of cultivated polyploids imply that significant advantages may be conferred by the presence of multiple genomes in the same nucleus. Possible explanations for this include greater heterozygosity leading to heterosis and buffering against deleterious alleles (Mendiburo and Peloquin 1977; Sanford 1983), increased allelic diversity, greater multiplicity of multimeric enzyme forms (Soltis and Soltis 1993), introduction of alleles heretofore unselected or subjected to different selection pressures in diploids (Jiang *et al.* 1998), and association of polyploidy with capability for vegetative reproduction, perenniality, and ability to colonize new niches (Stebbins 1950). Polyploid formation usually results in speciation, with reproductive isolation from progenitors and allied species. The effects of domestication superimposed on polyploid formation have greatly narrowed the genetic diversity in the gene pools of many major crops. In such cases, expanding the germplasm base is essential for overcoming the many challenges facing producers, processors, and consumers.

Attempts to introduce variability from wild diploid species into polyploids have taken several paths and have frequently met with difficulty. Many attempts have been made to hybridize diploid and tetraploid species directly, creating unstable genotypes that are difficult to maintain. Other avenues for introgression have included somatic doubling of a diploid followed by crossing with a tetraploid, diploid by tetraploid crosses using $2n$ gametes, or formation of triploids followed by doubling to hexaploids and elimination of chromosomes either spontaneously or through repeated backcrossing to the tetraploid. In less common instances, artificial amphidiploids have been produced and crossed with the tetraploid cultigen. Transmission genetics have been evaluated cytologically for several species (Stebbins 1950), but molecular-level studies have been possible only recently (Song and Osborn 1995; Liu *et al.* 1998).

In this article, genetics of introgression from a synthetic amphidiploid into cultivated peanut are reported. Unlike many other natural polyploid species for which multiple polyploidization events have been identified and that permit exchange of moderate levels of genetic variability among polyploids (Soltis and Soltis 1993, 2000), the cultivated tetraploid ($2n = 4x = 40$) *Arachis*

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hypogaea is believed to have originated recently from a single hybridization event (Kochert *et al.* 1996). Archaeological evidence from excavations in Peru place the origin of *A. hypogaea* at least 3500 years ago (Singh and Simpson 1994). DNA-level variability is so limited that attempts to identify polymorphism among varieties and landraces using restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD) markers (Halward *et al.* 1991; Kochert *et al.* 1991) have failed. Among >70 simple sequence repeat (SSR) loci evaluated, only 6 showed DNA polymorphism among *A. hypogaea* accessions (Hopkins *et al.* 1999).

Despite the paucity of DNA-level variability among cultivated genotypes, a wealth of diversity exists in other Arachis species. In the genus Arachis section Arachis, there are 27 species representing three genomes, A, B, and D. Additionally, 42 congeners have been identified in the other eight sections that comprise the Arachis genus (Krapovickas and Gregory 1994). Only one other species in section Arachis, *A. monticola*, is tetraploid and readily crossable with *A. hypogaea*, but *A. monticola* was indistinguishable from *A. hypogaea* based on DNA markers (Halward *et al.* 1991; Kochert *et al.* 1991).

In contrast to some botanical families, interploid crosses are not prominent among major legume species. Phaseolus and Vigna are diploid, and although Glycine ($2n = 40$) shows evidence of being an ancient polyploid (Hadley and Hymowitz 1973; Shoemaker *et al.* 1995), crosses to $2n = 20$ material are rare. Only in the autopolyploid *Medicago sativa* have interspecific crosses been used to an appreciable extent (Bingham 1980).

Recently, a synthetic amphidiploid, TxAG-6 (Simpson 1991; Simpson *et al.* 1993), was developed and has been used to introduce root-knot nematode resistance into cultivated peanut (Burow *et al.* 1996; Simpson and Starr 2001). TxAG-6 was developed through the cross [*A. batizocoi* × (*A. cardenasii* × *A. diogoi*)]$^{4\times}$, where *A. cardenasii* and *A. diogoi* are A-genome diploids, and *A. batizocoi* was considered to be a B-genome diploid ancestor (Husted 1933, 1936; Smartt *et al.* 1978b). This cross has made possible the generation of the first molecular map representing the entire tetraploid genome of peanut as well as study of the transmission genetics of chromatin from a synthetic amphidiploid into cultivated peanut.

MATERIALS AND METHODS

Plant material: Peanut BC_1 lines were generated using the tetraploid ($2n = 4x = 40$) breeding line TxAG-6 (Simpson *et al.* 1993; Burow *et al.* 1996) as donor parent and the cultivated *A. hypogaea* ($2n = 4x = 40$) variety Florunner as recurrent parent (Figure 1). Seventy-eight BC_1 plants were used as the mapping population. Diploid progenitors of TxAG-6, used as standards on Southern blots, were K9484 (P.I. 338312) *A. batizocoi* Krapov. & W. C. Gregory, GKP10017 (P.I. 262141) *A. cardenasii* Krapov. & W. C. Gregory, and GKP10602 (P.I. 276235) *A. diogoi* Hoehne. For classification see Krapovickas and Gregory (1994).

DNA extraction: Young leaves were collected, divided into 2-g samples, frozen in liquid nitrogen, and stored at −80° until use. Samples were ground in liquid nitrogen, using a mortar and pestle until powdered finely, then dissolved in 20 ml of extraction buffer [0.050 m citric acid, 0.50 m glucose, 0.010 m Na_2EDTA, 2.0% (w/v) polyvinylpyrrolidone-40 (PVP-40), 5.0% (v/v) Triton X-100, 0.25% (w/v) spermidine, titrated to pH 5.0 with NaOH] with freshly added antioxidants [0.1% (w/v) ascorbic acid, 0.2% (v/v) 2-mercaptoethanol, 0.1% (w/v) disodium diethyldithiocarbamate (Na_2Et_2dtc), and 0.4% (w/v) $NaHSO_3$], shaken on ice for 10 min until dissolved, and filtered through four layers of cheesecloth. Filtrates were centrifuged for 20 min at 2800 × g to sediment nuclei and unbroken cells. Pellets were homogenized for 30 sec in 20 ml of clearing solution [absolute ethanol containing 0.167 m acetic acid, 1.0% PVP-40, 0.5% ultrapure sodium dodecyl sulfate (SDS), titrated to pH 5.5 with NaOH, plus antioxidants except $NaHSO_3$], using a motorized tissue homogenizer at 11,500 rpm, then heated at 65° for 20 min and centrifuged. Pelleted nuclei were broken by vortexing briefly in 10 ml of lysis buffer [0.050 m citric acid, 0.14 m NaCl, 0.050 m Na_2EDTA, 2.0% (w/v) PVP-40, and 2.0% (w/v) ultrapure SDS, titrated to pH 5.0 with NaOH, autoclaved, antioxidants added freshly] and heating at 65° for 20 min. After centrifugation, 3.5 ml of 5 m KOAc pH 5.2 was added, solutions were incubated on ice for 30 min and centrifuged, and DNA was precipitated with 7 ml of 2-propanol. After centrifugation, pellets were washed with 10 ml of 70% ethanol, air dried, and resuspended in 4 ml of TE. One milliliter of 5× TNE (0.5 m TrisHCl, 1.5 m NaCl, and 0.025 m Na_2EDTA, pH 8.0) plus 50 μg of RNase A (Sigma, St. Louis) were added to each sample, samples were incubated at 37° for 30 min, then extracted once with phenol-chloroform and twice with chloroform, and precipitated with 10 ml of ethanol. Air-dried samples were resuspended in 200 μl of TE and stored at −20°.

Mapping: DNA samples (2 μg each) were digested with *Eco*RI or *Hin*dIII, separated electrophoretically on 0.8% agarose gels at 0.5 VDC/cm for ~24–30 hr, and transferred to Hybond N+ (Amersham-Pharmacia, Piscataway, NJ) membrane in 0.4 m NaOH. Probes used were cDNAs isolated from root- and shoot-derived cDNA libraries graciously provided by Dr. Gary Kochert, University of Georgia. Previously mapped *Vigna* cDNA clones, kindly provided by Dr. Nevin Young, University of Minnesota, were also used. Random hexamer labeling was performed using 50 ng of template for primer extension by Klenow fragment, using 25 μCi of [α-^{32}P]dCTP (6000 Ci/mmol; Chittenden *et al.* 1994). Blots were hybridized at 65° for 36 hr in hybridization solution (Chittenden *et al.* 1994) containing 0.5% dextran sulfate, washed at 65° in 0.3× SSC/0.1% SDS final stringency, and exposed to Kodak XRP film or equivalent.

Linkage analysis: Linkage analysis was performed using MAPMAKER/EXP v 3.0 for DOS/386 and for Solaris (Lander *et al.* 1987). Analysis was performed using double crossover detection. Trios of markers that exhibited significant numbers of double crossovers relative to the expected frequency had members removed from map distance calculations (and shown in parentheses instead) to eliminate inflation of recombinational distances. Markers were considered linked if the calculated recombination distance was <30 Kosambi cM, with a LOD score of >4.0. Subgenomic origins of individual markers were determined by comparison to the alleles present in the diploid progenitors (see Figure 2; Reinisch *et al.* 1994); the inferred diploid origin of each allele was indicated on the map. A linkage group segment was considered to be derived

from a particular ancestor if two or more adjacent markers were of unambiguous origin; the backbone of each linkage group is filled in color to illustrate this (Figure 3).

Cotransfer of linked nonhomeologous marker loci: The probability of ancestral cotransfer of linked nonhomeologous markers (including the intervening chromatin) was estimated as the probability of a segment of that length possessing no intervening comapping markers, on the basis of the frequency of detection of intervening loci among homeologous chromosomes. This was estimated as

$$\left(\frac{a_L}{h_L}\right)^{d_L/h_L} \cdot f(d_S),$$

where d_L is the larger of the distances separating the two linked markers in the two linkage groups where the markers were located, a_L is the mean spacing between markers in the corresponding genome L, h_L is the mean spacing between mapped markers in genome L that have homeologous counterparts, and $f(d_S)$ is a correction factor for the smaller of the two distances,

$$\left(\frac{a_S}{h_S}\right)^{d_S/h_S},$$

where $a_S/h_L > 1.0$, and 1.0 where $a_S/h_L \leq 1.0$. In practice, (a/h) was found to be $\sim\frac{1}{2}$ for A and B genomes. For permutations, the probability of a given combination of cotransfers was the product of the independent probabilities of each cotransfer.

RESULTS

Polymorphism, segregation, and recombination: *Polymorphism among parents:* The high degree of DNA polymorphism observed between cultivated and wild species in a previous report (Burow *et al.* 1996) was used as the basis for a systematic attempt to map genetic variability in cultivated peanut. Examination of the parents of the synthetic polyploid TxAG-6 by RAPD analysis had indicated 66% polymorphism between the three wild species (*A. cardenasii*, *A. diogoi*, and *A. batizocoi*) and Florunner (M. Burow, unpublished results). A high level of DNA polymorphism was observed in the present report between the cultivated peanut variety Florunner and the synthetic polyploid TxAG-6 (Figure 1). Eighty-three percent polymorphism was observed on a per-band basis between Florunner and TxAG-6, using RFLP data (Table 1).

Two hundred twenty cDNA probes were used to map 370 RFLP loci onto 23 linkage groups using a BC_1 mapping population. A total of 917 bands were observed, for an average of 4.1 bands per probe. A mean of 1.68 loci per probe were mapped. The total length of the tetraploid map, 2210 cM, was slightly greater than twice the length (1063 cM) of the diploid map (Garcia *et al.* 1995).

Segregation distortion: Twenty-five percent of the markers mapped showed significant deviation from the expected 1:1 allelic segregation pattern for BC_1 progeny (Table 2, Figure 3). Because of the high frequency of expected false positives (19) in 370 comparisons at the

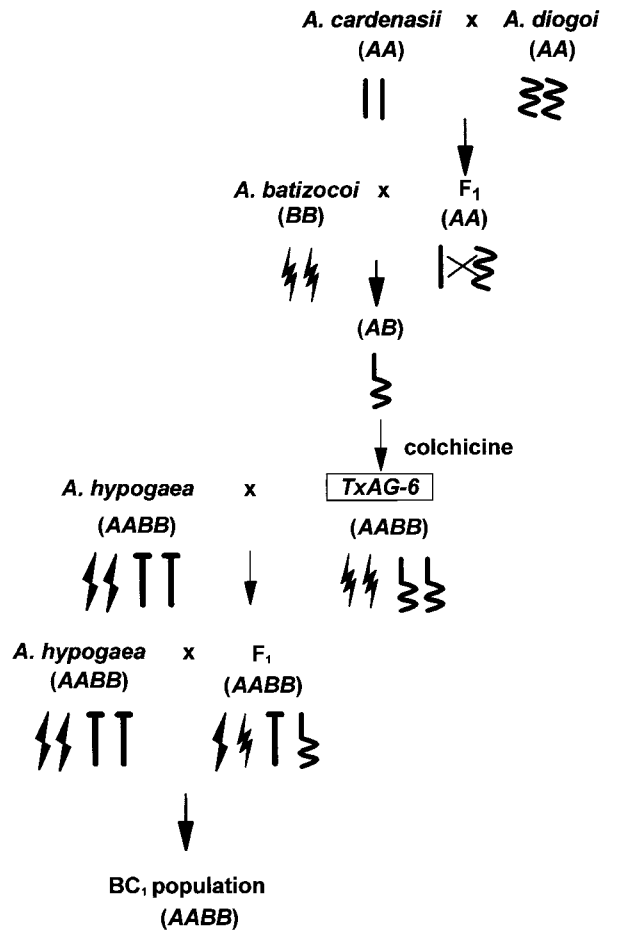

Figure 1.—Crossing scheme used to generate the mapping population. A schematic of a single chromosome pair is drawn to illustrate the expected location of recombination observed between the A-genome chromosomes of *A. cardenasii* and *A. diogoi*. Recombination between chromosomes in the F_1 is indicated by ×.

$P = 0.05$ level of probability, the conservative Bonferroni (p/r) level of probability (Snedecor and Cochran 1980), where r is the number of comparisons made, was used to evaluate the statistical significance of these deviations. Using this statistic, proportions of from 40

TABLE 1

Polymorphism among peanut species

Genotype[a]	H	B	C	D
B	109			
C	190	108		
D	138	114	169	
T	155	490	233	212

Data were taken using probes that were mapped. Data are given as the no. of bands held in common by each pair of accessions. A total of 917 bands were scored.

[a] H, *Arachis hypogaea* cv. Florunner; B, *A. batizocoi* K9484; C, *A. cardenasii* GKP10017; D, *A. diogoi* GKP10602; T, TxAG-6.

TABLE 2

Length, species origins, and segregation distortion in tetraploid peanut linkage groups

	Length (cM)				No. of markers						Seg. dist. mkrs.		
LG	Tot.	A. bat	A. car	A. dio	Tot.	A. bat	A. car	A. dio	Unk.	Unex.	Pct.	*N*+	*N*−
1	164.6	0.0	145.7	18.9	27	0	25	2	0	0	22	2	4
11	135.0	135.0	0.0	0.0	27	25	0	0	2	0	4	1	0
3	169.2	0.0	54.3	114.9	15	0	3	6	3	3	40	2	4
13	74.6	74.6	0.0	0.0	23	22	0	0	1	0	48	11	0
6	91.9	0.0	36.6	55.4	15	0	5	9	0	1	27	4	0
16	102.1	102.1	0.0	0.0	16	16	0	0	0	0	31	5	0
5	69.7	0.0	69.7	0.0	8	0	8	0	0	0	25	0	2
15	125.6	125.6	0.0	0.0	16	16	0	0	0	0	25	1	3
20	74.7	74.7	0.0	0.0	18	18	0	0	0	0	78	14	0
10	34.4	0.0	0.0	33.5	6	0	0	5	1	1	17	1	0
14	93.6	93.6	0.0	0.0	22	22	0	0	0	0	14	2	1
4	107.4	0.0	0.0	107.4	17	0	0	16	0	1	0	0	0
17	75.1	75.1	0.0	0.0	12	12	0	0	0	0	42	1	4
7	79.9	0.0	0.0	79.9	13	0	0	8	3	2	23	3	0
19	84.3	84.3	0.0	0.0	18	18	0	0	0	0	50	1	8
9.1	45.0	0.0	16.6	28.4	8	0	2	5	0	1	25	1	1
9.2	103.1	0.0	103.1	0.0	15	0	13	0	2	0	0	0	0
18	65.0	65.0	0.0	0.0	21	21	0	0	0	0	19	3	1
8	67.5	0.0	0.0	67.5	9	0	0	8	0	1	56	4	1
12	110.8	110.8	0.0	0.0	32	32	0	0	0	0	3	1	0
2	165.9	0.0	165.9	0.0	22	0	15	0	4	3	5	1	1
21	140.0	0.0	0.0	0.0	8	4	1	1	2	0	63	5	0
22	2.0	0.0	2.0	0.0	2	0	2	0	0	0	0	0	0
Sum	2207	941	619	506	370	206	74	60	17	13	94	64	30

Tot., total; A. bat., *A. batizocoi*; A. car., *A. cardenasii*; A. dio., *A. diogoi*; Unk., unknown species origin; Unex., unexpected because species origin differed from that of flanking markers; Pct., percentage; Seg. dist. mkrs., markers with segregation distortion. *N*+ indicates the number of markers for which a significant ($P < 0.05$) excess of the allele derived from the donor parent (TxAG-6). *N*− indicates the number of markers for which a significant ($P < 0.05$) deficiency of the allele derived from the donor parent (TxAG-6).

to 60% donor parent-derived alleles were expected; a range of from 25 to 86% was observed. Unexpectedly, 64 of the 94 loci with distorted segregation ratios had an excess of alleles from the synthetic amphidiploid donor parent; these were distributed as 10 groups, each consisting of at least 2 linked markers with distorted segregation. Thirty markers had an excess from the cultivated recurrent parent; these were present as 7 linked groups. Overall, in 4 linkage groups, ≥50% of the markers demonstrated segregation distortion; in 3 of the 4, there was a consistent pattern of favoring the allele derived from the synthetic amphidiploid. In a further 6 linkage groups, between 25.0 and 49.9% of the markers' segregation ratios fell outside the expected range. In only 6 linkage groups were there <10% of loci with distorted segregation.

Recombination: *Species origin of markers:* Origins of segregating markers were determined by comparison of mobility to parental DNA fragments included on each Southern blot (Figure 2A). The largest number of markers was derived from *A. batizocoi* (206 markers, 56% of total; Table 2), with the remainder from *A. cardenasii* (20%) and *A. diogoi* (16%). An additional 3% of the alleles were recognized as derived from an ancestor to TxAG-6 but were not in accord with the species designation of flanking markers. Five percent of segregating marker alleles did not correspond to any of the three diploid ancestors, TxAG-6, or Florunner (Figure 2B). Excluding these two sets of markers, *A. batizocoi* comprised 61% of the markers mapped, and the A-genome diploids, 39%. Ignoring differences in detection of polymorphism, assuming that the chromosomes of *A. batizocoi* paired with the B genome of *A. hypogaea*, and that chromosomes derived from *A. cardenasii* and *A. diogoi* paired with the A genome of cultivated peanut (Gregory and Gregory 1976; Smartt *et al.* 1978b; Singh and Moss 1982; Figure 1), the expected number of markers derived from the three species would be 0.50:0.25:0.25, respectively. Taking into account differences in detection of polymorphism between species (Table 1), the percentage contribution of *A. batizocoi* to the total number of mapped markers was in inverse proportion to the degree of similarity to the *A. hypogaea* parent; however, this was not so with *A. cardenasii* and *A. diogoi*. The expected number of markers contributed by *A. batizocoi* was calculated as

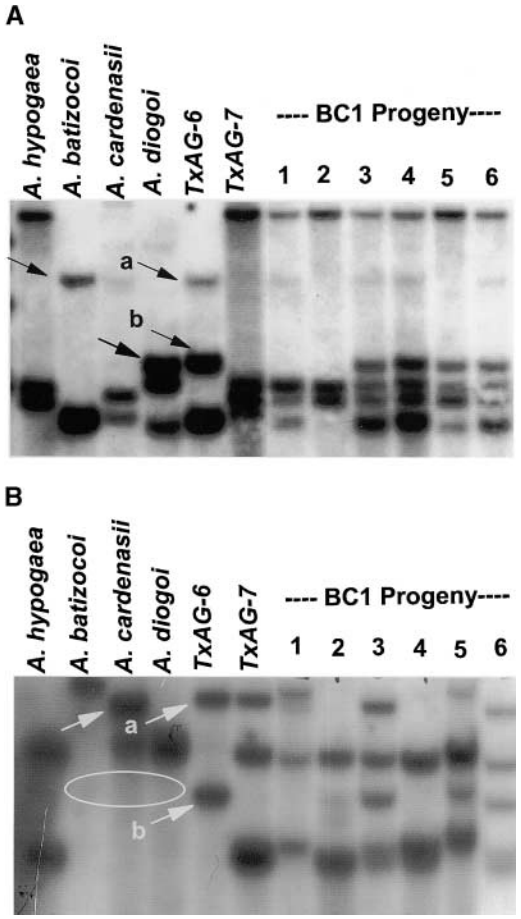

FIGURE 2.—Identification of species origin of markers. (A) Origin of markers generated using probe R193. Arrows denote markers present in TxAG-6 but not in *A. hypogaea*. BC1, individual BC_1 plants. Marker a was derived from *A. batizocoi* and marker b from *A. diogoi*. (B) Example of one marker without an apparent origin. Marker a was derived from *A. cardenasii*, but marker b was present only in TxAG-6, not any of the three diploid ancestors (the location of the expected bands is marked by an oval). The probe used was R2609.

$$N\left[\frac{\text{sim(HB)}}{\text{sim(HB)} + 0.5[\text{sim(HC)} + \text{sim(HD)}]}\right]^{-1},$$

where N is the total number of markers mapped, sim (x, y) is the number of markers present in both genotypes, H is *A. hypogaea*, B is *A. batizocoi*, C is *A. cardenasii*, and D is *A. diogoi*. This assumes that one-half of the total genetic contribution was derived from *A. batizocoi*, and the similarity coefficients of *A. cardenasii* and *A. diogoi* were averaged because they would be expected to contribute one-half of the genome combined. The expected proportion of *A. batizocoi*-derived markers was 0.60, similar to the observed 0.61. For *A. cardenasii* and *A. diogoi* the expected contributions were 0.17 and 0.23, respectively; however, the observed values were almost the reverse: 0.22 and 0.18. This is explained as the percentage of genetic contribution of the two A-genome diploids being affected by the chance composition of recombinant chromosomes in a single gamete from the (*A. cardenasii* × *A. diogoi*) F_1. Three linkage groups each were derived entirely from *A. cardenasii* and *A. diogoi*, and the total lengths of these LGs were 380 *vs.* 278 cM, respectively.

Recombinational lengths of linkage groups: Relative recombination lengths of chromosomes derived from each diploid progenitor species of TxAG-6 did not indicate differences in pairing affinity between *A. hypogaea* and the diploid species. For comparison, terminal markers linked at LOD scores of <4 were omitted; likewise omitted was LG21, which is of uncertain origin (see below). Relative species contributions to the total recombinational length were 0.46:0.29:0.25 for *A. batizocoi*, *A. cardenasii*, and *A. diogoi*, respectively. Assuming proportionality between physical and genetic distances, using published optical measurements of the species' total chromosomal lengths (FERNANDEZ and KRAPOVICKAS 1994), and averaging the contributions of *A. cardenasii* and *A. diogoi* initially to compute the *A. batizocoi* ratio, the expected ratios would be 0.47:0.28:0.25, values that were very close to the observed data.

Mosaic composition of the A genome: As expected on the basis of the pedigree (Figure 1), the A genome in TxAG-6 was composed of chromatin from two species (Figure 3). Three linkage groups (LG2, LG5, and LG9.2) were derived entirely from *A. cardenasii*, three (LG4, LG7, and LG8) from *A. diogoi*, four (LG1, LG3, LG6, and LG9.1) from both species, and two (LG10, one end could be derived from *A. cardenasii* or *A. diogoi*, and LG21, see below) were uncertain. The mosaic chromosomes composed of chromatin from *A. cardenasii* and *A. diogoi* presumably were the result of meiotic crossing over in the (*A. cardenasii* × *A. diogoi*) hybrid prior to crossing with *A. batizocoi*. This crossing over could not have occurred in TxAG-6, which was maintained vegetatively, and recombination between *A. cardenasii*- and *A. diogoi*-derived chromosomes was unlikely to have occurred after hybridization of the (Florunner × TxAG-6) F_1 with TxAG-6.

Salient features of linkage groups: Twenty-three linkage groups are present in the map; these were composed of nine pairs of homeologous linkage groups, one trio representing a homeologous chromosome pair, one fragment consisting of two markers, and 1 linkage group that was possibly an artifact (Figure 3). Given that cultivated peanut is a disomic polyploid ($2n = 4x = 40$), 20 linkage groups were expected.

The linkage group pair LG1/LG11 illustrates key points common to many linkage groups. LG1 was one of the two longest linkage groups and together with LG11 constituted the longest LG pair. LG1 consisted of 27 markers, with a length of 165 cM. The majority was derived from *A. cardenasii*, with one end derived from *A. diogoi*. LG11 is identified by 27 probes for a total length of 135 cM and is composed solely of markers derived from *A. batizocoi*. Markers from 15 probes de-

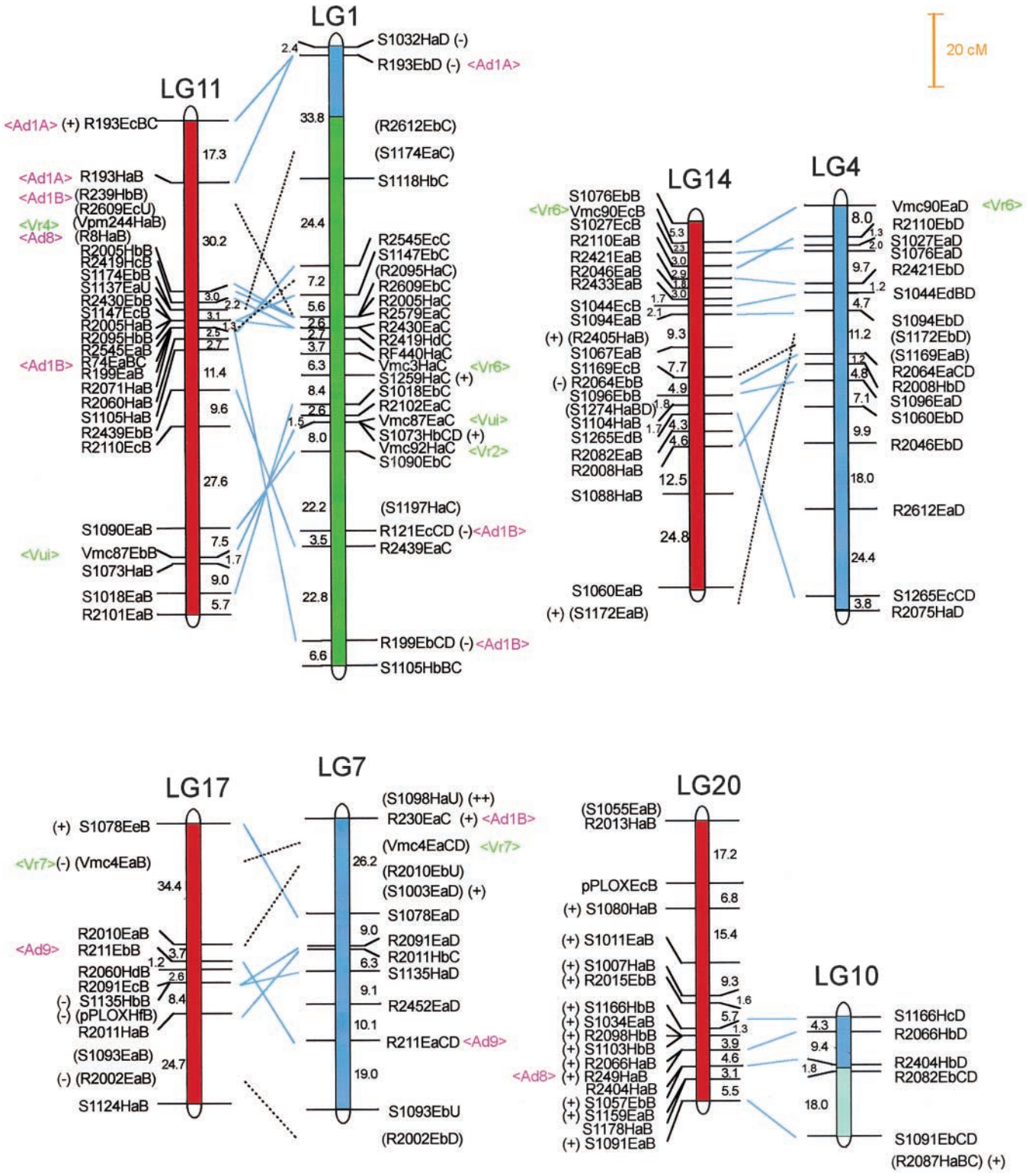

FIGURE 3.—RFLP marker map derived from the BC_1 mapping population. Map distances are given in Kosambi centimorgans. Blue lines connect markers labeled by the same probe, which can be identified on homeologous linkage groups. Dotted black lines connect marker pairs in which at least one marker was judged to fit poorly in the map because of a large number of double recombinants. Colors: solid red, *A. batizocoi*-derived region; solid green, *A. cardenasii*-derived region; solid blue, *A. diogoi*-derived region; aqua, species origin undetermined. Areas cross-hatched represent linkages between chromosome segments or markers at 4 > LOD > 3. Marker designations are interpreted as follows: prefix letter (R, peanut root; S, peanut shoot; V, *Vigna*; E, *Beta*); four-digit cDNA probe number; enzyme (E, *Eco*RI digest; H, *Hin*dIII digest); band designation (a, b, c, etc.—order among polymorphic bands from lowest to highest migration); origin (B, *A. batizocoi*; C, *A. cardenasii*; D, *A. diogoi*; U, unknown origin—not present in any parent). Markers with designations in parentheses denote markers placed approximately; accurate placement was not possible because of a high number of apparent double crossovers involving this marker. To some markers are appended pluses or minuses in parentheses. These specify the direction and degree of segregation distortion: (- -), 20–30% donor parent allele; (−), 30–40%; (+), 60–70%; (++), 70–80%; (+++), 80–90%. To the side of some markers is an additional designation in color. This refers to map location on other maps. Designations are interpreted as follows: origin (Vr, *Vigna radiata*; Vu, *Vigna unguiculata*; Ad, *Arachis* diploid map) and then linkage group designation.

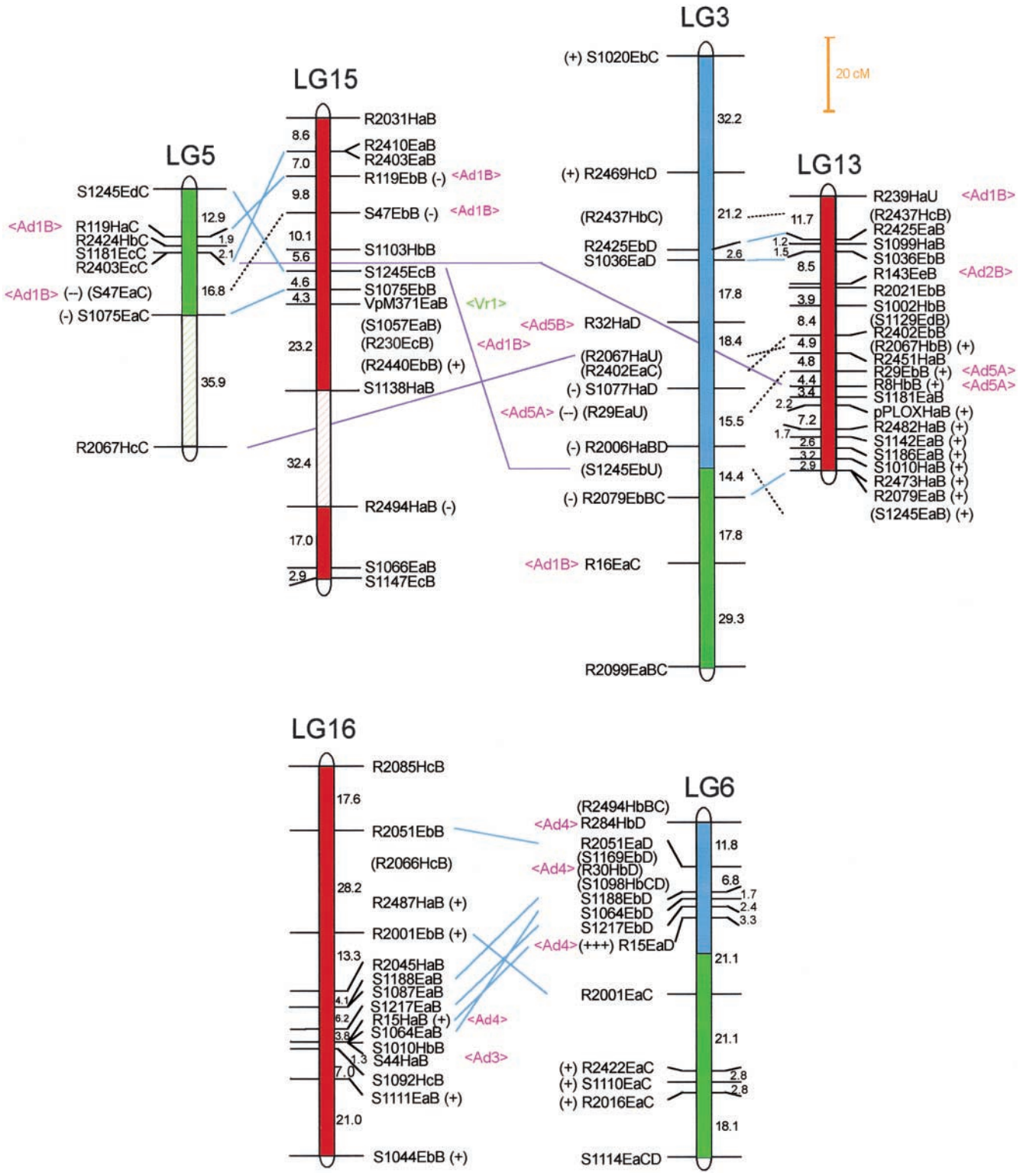

FIGURE 3.—*Continued.*

tected RFLPs on both LG1 and LG11. A major inversion encompassing about one-half of the chromosome differentiated LG1 from LG11, with the region delimited by R193 and R2430 being collinear between both linkage groups. Cloned RAPD marker RKN440, linked to root-knot nematode resistance (BUROW *et al.* 1996), was mapped onto LG1 and denoted RF440. Three other markers (S1137, R2545, and R2430) have also been associated with nematode resistance (CHOI *et al.* 1999).

LG21 was an exception to the presence of pairs of

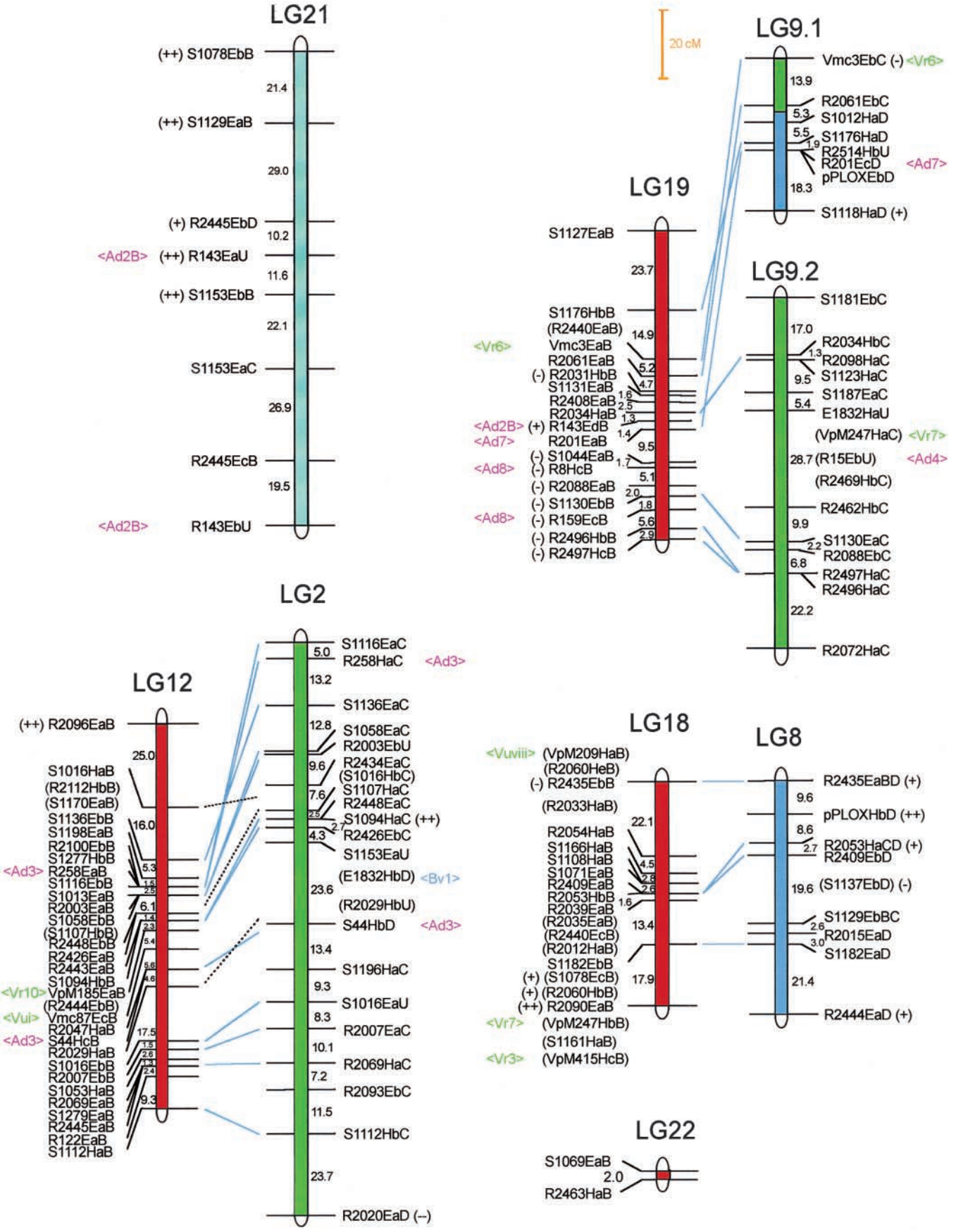

FIGURE 3.—*Continued.*

homeologous chromosomes. This linkage group was composed of only eight markers, including four from *A. batizocoi* and two from *A. cardenasii*. One possible explanation of the apparent mosaic composition of this chromosome is that this linkage group may have undergone recombination between genomes, presumably in TxAG-6. Alternatively, this linkage group may have been an artifact caused by pseudolinkage. The average spacing of markers (17.5 cM) was triple the average (5.7 cM) for the remainder of the genome, and there were many apparent double crossovers between trios of markers. Additionally, four of the eight markers had severe segregation distortion, with a proportion of from 0.71 to 0.79 of these four alleles originating from the synthetic amphidiploid. This distortion falsely increases the statistical significance of the association and may have resulted in apparent association of markers that are actually unlinked.

One trio of linkage groups involves LG19, LG9.1, and LG9.2. A single homeolog could not be constructed to LG19. Rather, two partial homeologs (LG9.1 and LG9.2) have been identified. There were probes that map to both halves of LG9.2 as well as LG19, but the large gap in LG9.2 was not reflected in LG19.

Regions of unusual composition were present in several linkage groups. The first type of event was the presence of markers of undetermined origin, which occurred on LG11 (one marker), LG7 (two markers), LG9.2 (two markers), LG3 (three markers), and LG2 (four markers). These markers could not be identified in any of the parents (Florunner, TxAG-6, and the three wild diploid species parental to TxAG-6). A second type of unusual event involved 3% (13 in number) of the total markers (excluding LG21); these were present in regions where flanking markers were both derived from a different species. In 12 cases, markers from the two A-genome diploids were interspersed. One-half of these markers occurred in two linkage groups. In LG2, E1832HbD and S44HbD may indicate a region derived from *A. diogoi*, and the terminal marker R2020 may have been inherited from *A. diogoi* likewise. Neither were counted as *A. diogoi*-derived regions; in the case of E1832HbD and S44HbD, E1832HbD could not be mapped precisely, and R2020EaD was one sole marker—not enough to define a region. In LG3, two of the three markers were ones that could not be mapped accurately due to apparent multiple double crossovers, and it is possible that these markers may be more closely associated than it appears. In the other instances, these markers may represent relatively small double crossovers that occurred between *A. cardenasii* and *A. diogoi* in the diploid F_1 produced by their cross.

Apparent absences of large chromosome segments or series of markers occurred in four linkage group pairs (LG6/LG16, LG10/LG20, LG5/LG15, and LG3/LG13). This appeared to be artifactual in one case but not in the others. For the LG pair LG3/LG13, the extra chromosome segments at the ends of LG3 included markers judged to be less reliable in scoring because of their faint appearance. This was not the case for the other three linkage group pairs, where markers at the ends were deemed to be of good signal strength. Additionally, markers in these regions demonstrated generally increasing map distances from other markers, not lower recombination distances with interior markers, which would be characteristic of poor quality markers mapped to the ends of linkage groups. For the LG5/LG15 pair, the extra markers consisted of a group of three that were associated with the remainder of the linkage group by a maximum LOD score (3.95) less than the 4.0 statistical threshold used elsewhere. Failure to detect DNA polymorphism in certain homeologous regions appeared to be the most likely explanation for missing segments for several reasons:

1. There was no evidence for duplication of markers on the longer member of each linkage group pair.
2. Data suggested that the missing segments in these linkage group pairs were not the result of physical deletions of chromatin. In particular, in LG6, LG13, LG15, and LG20, there was no significant difference in the mean number of bands between pairs of chromosome segments in which polymorphic loci were absent in one member *vs.* in adjacent segments. The mean numbers of bands detected were 4.5 per probe in *A. hypogaea*, 5.5 in TxAG-6, and 7.5 in the BC_1 progeny.
3. There was some evidence suggesting a lack of polymorphism in the missing chromosome segments. Specifically, there was a statistically significant reduction from 2.7 to 2.1 polymorphic markers per probe distinguishing TxAG-6 from *A. hypogaea* in the regions of LG6, LG13, LG15, and LG20 where no homeolog was detected relative to adjacent segments where both homeologs were observed. Only in one case, that of S1169EaB on LG4, was there evidence for intergenomic recombination.

Comparative data: *Duplication of markers and synteny among nonhomeologous chromosomes:* Southern hybridization analysis revealed a large number of probes highlighting multiple bands (Table 3), some of these suggesting possible duplication of linkage blocks. Eighty-nine probes produced markers on both homeologous linkage group pair members. In addition, 45, 25, 8, and 1 probes segregated at two, three, four, and five nonhomeologous loci, respectively. These 45 probes comprised 124 bands (Table 3). It should be noted that some probes hybridized to both homeologous and nonhomeologous linkage groups.

There were nine pairs of linked groups of markers occurring on nonhomeologous chromosomes. There was one case in which three probes (all of which were confirmed to have different hybridization patterns) were shared between two nonhomeologous linkage groups.

TABLE 3

Markers in common to pairs of linkage groups

LG	1	11	3	13	6	16	5	15	14	4	20	10	17	7	19	9.1	9.2	18	8	12	2	21	22
1	27	*16*																					
11	*16*	27		2																			
3			15	*8*			2																
13		2	*8*	23			3								2							2	
6					15	*6*																	
16					*6*	15																	
5	2	3					8	*5*															
15							*5*	16			2			2									
14									22	*12*													
4									*12*	17													
20								2			18	*4*											
10											*4*	6											
17													12	*8*				2					
7								2					*8*	13									
19				2											18	*4*	*4*						
9.1															*4*	7							
9.2															*4*		15						
18												2						21	*4*				
8																		*4*	9				
12																				32	*15*		
2																				*15*	24		
21				2																		8	
22																							2

Numbers represent no. of linked markers in common between the given pair of linkage groups. The total no. of markers in a linkage group is underlined. Markers common to homeologous chromosomes are in italics. Linked markers common to nonhomeologous chromosomes are in roman type.

TABLE 4

Probabilities of co-inheritance of duplicated nonhomeologous marker pairs

Marker pair	LG	Genome	Dist. (cM)[a]	Prob.[b]
R2031/R2440	15	B	61.6	
	19	A	17.4	0.01
S1057/S1103	15	B	25.1	
	20	B	8.5	0.18
S1245/S1181	13	B	28.1	
	5	A	16.9	0.15
S1181/R2067	13	B	15.1	
	5	A	34.8	0.21
R143/S1129	13	B	10.1	
	21	(B)	39.2	0.07
R0008/R0239	13	B	51.3	
	11	B	0.0	0.03
R0008/R0143	13	B	28.4	
	19	B	12.6	0.14
R2060/S1078	18	B	65.9	
	17	B	39.3	<0.01
R2067/S1245	3	A	31.9	
	5	A	51.7	0.10

[a] The distance between the pair of markers on the linkage group.

[b] The probability that the two markers could have been co-inherited in a single transfer event from one chromosome to the other without intervening markers common to both regions being identified.

Three probes (S1245, S1181, and R2067) mapped to both LG5 and LG13, and the three marker pairs were collinear. For S1245/S1181 and S1181/R2067, the probabilities that duplication of each marker pair occurred as a single cotransfer of both marker loci (and the intervening chromatin) were estimated as 0.15 and 0.21 (Table 4)—both were considered highly possible. S1245 and R2067 were also common to LG5 and LG3, and probe S1245 mapped to LG3, LG13, and LG15. On the basis of the number of markers in common, evidence suggested that LG5/LG15, LG3/LG13, and LG6/LG16 were the most likely homeologous grouping of these linkage groups. LG5 was considered homeologous to LG15, by virtue of having the most probes (five) in common, four of them collinear. All markers on LG5 were present solely in the *A. cardenasii* parent. Consistent with potential homeology, LG15 was derived entirely from *A. batizocoi*. This was also more in accord with delimitation of linkage groups in the diploid cross (see below).

There were seven additional pairs of nonhomeologous markers shared between two linkage groups (Table 4). Four were considered reasonably possible to have each resulted from a single chromatin transfer event to a nonhomeologous chromosome ($P > 0.05$), but pairwise distances on one linkage group for the three other marker pairs were >40 cM and probabilities of cotransfer were lower. Calculation of the probabilities of all possible permutations of occurrence indicated that the probabilities of zero, one, two, and three marker pairs being co-inherited in duplication or translocation events were 0.38, 0.40, 0.17, and 0.04, respectively.

Comparison of the tetraploid and diploid peanut maps: Results from the tetraploid map of *A. hypogaea* were compared to a 115-marker RFLP map made from a cross between two wild peanut species (*A. cardenasii* × *A. stenosperma*; Halward *et al.* 1993), which was extended to *A. hypogaea* by mapping the $F_{10}C_9$ progeny *A. cardenasii* × *A. hypogaea* (Garcia *et al.* 1995). In that cross, 11 linkage groups representing only the A genome of *A. hypogaea* were identified. Twenty-three cDNAs mapped as RFLPs on the diploid map (*A. cardenasii* × *A. stenosperma*; Halward *et al.* 1993) were hybridized to the tetraploid mapping population, and 5 sets of corresponding linkage groups were identified. The greatest number of probes common to 2 different linkage groups was five probes mapping both to the tetraploid LG1 and *Ad1a/Ad1b* (*A*rachis *d*iploid *LG1*a/*1b*). Two probes (R121 and R199) mapping to the tetraploid LG1 were placed on *Ad1b*. Two probes (R239 and R74) mapping to LG11 were also placed on *Ad1b*, giving further evidence for the homology of LG1 and *Ad1b*. The maps of LG1 and LG11 also included marker R193, mapped on *Ad1a*. In the tetraploid data set, there was strong evidence (LOD > 4.0) for the linkage of R193 and the rest of LG1. There was, however, a major difference between the tetraploid and diploid maps regarding *Ad1b*. LG1 and LG11 lacked markers corresponding to approximately one-third of *Ad1b*, probes S47 and R119 (mapping to *Ad1b*) were mapped to LG5 and to its homeolog LG15, and R230 mapped to LG15. There was no statistical evidence (LOD ≥ 2, distance ≤40 cM) for linkage of markers mapping to LG1 and LG5. Given that *Ad1* and LG1 were both mapped in crosses in which one parent was *A. cardenasii* or was derived from *A. cardenasii*, the difference between maps may be based on statistical interpretation of the data. However, as translocations among *A. hypogaea* varieties have been reported (Wynne and Halward 1989), the possibility of a major rearrangement cannot be discounted.

There was also evidence, based on a limited number of markers, for further associations between maps. These were as follows:

1. Three probes producing markers on the *A. diogoi*-derived segment of LG6 mapped to *Ad4*, as did one marker on the homeolog LG16. However, one additional probe on LG16 mapped to *Ad3*.
2. Two markers (R258HaC and S44HbD) on LG12 and two (R258EaB and S44HcB) on LG2 were produced by probes mapping onto *Ad3*.
3. The central region of LG3 had two markers (R32HaD and R29EaU) that were associated with *Ad5a* or *Ad5b*. The homeologous region of LG13 also had two markers (R29EbB and R8HbB) placed on *Ad5a*. However,

other probes at both ends of LG3 or LG13 mapped to other diploid linkage groups but without a clear pattern.

4. Markers R8 and R159 in LG19 indicated correspondence of one region of LG19 to *Ad8*, but markers in other regions of LG19, as well as in LG9.1 and LG9.2, did not indicate a consistent correspondence.

Comparison of the tetraploid map to that of Vigna spp: Eleven cDNAs mapped previously on *Vigna radiata* and *V. unguiculata* (Menancio-Hautea *et al.* 1993) were mapped on peanut, producing 17 loci. On the basis of these limited results, it was not possible to determine synteny between Vigna and Arachis. The largest number of Vigna markers that mapped to a single peanut chromosome pair occurred on LG1 and LG11, where four Vigna probes were mapped (Figure 3). None of the four markers were derived from the same *V. radiata*/*V. unguiculata* linkage group or homolog, suggesting that there may be a substantial number of rearrangements distinguishing Arachis and Vigna.

DISCUSSION

A synthetic amphidiploid was used as a means to enhance allelic diversity in a species possessing limited variability due to recent polyploidization and barriers to interspecific gene transfer. Results from this study included (1) simultaneous introgression of chromatin from three wild diploid species into the two genomes of cultivated peanut, (2) development of the first tetraploid molecular map of peanut, (3) study of transmission genetics of this complex cross and its relation to the species origin of peanut, and (4) molecular marker-level identification of gene rearrangements that may be associated with reproductive isolation of peanut species from one another.

Introduction of variability into cultivated peanut: Introduction of chromatin from wild species using an amphidiploid (Simpson *et al.* 1993) was pursued for reasons of temporal efficiency and genome composition. In somatic doubling of the sterile three-way diploid progeny, a single event was needed to arrive at a 40-chromosome tetraploid instead of the multiple generations needed to obtain euploid (tetraploid) progeny by the hexaploid route. In addition, it was considered unlikely that a cross of an autotetraploid to *A. hypogaea* would allow proper pairing of A- and B-genome chromosomes (Smartt *et al.* 1978a; Singh and Moss 1982; Singh 1985). In practice, the amphidiploid parent of the mapping population [*A. batizocoi* × (*A. cardenasii* × *A. diogoi*)]$^{4\times}$ contained two genomes (A and B) from three distinct wild species.

Transmission genetics and ancestry of peanut: *Chromosome pairing and fertility:* Based on chromosome pairing of BC_1 progeny and on fertility of their hybrids, the combination of TxAG-6 and *A. hypogaea* appeared to fit the definition of a genome allopolyploid (Stebbins 1950; Rieger *et al.* 1976; Gaut and Doebley 1997). The tetraploid map indicated disomic inheritance in all linkage groups except LG21 and the fragmental LG22, because markers derived from *A. batizocoi* did not map to linkage groups composed of markers from *A. cardenasii* and/or *A. diogoi*. By comparison, cytological studies of *A. hypogaea* observed 20 chromosome bivalents at meiosis in 88–98% of cells; the exceptions were rare univalents, trivalents, and quadrivalents, which suggested limited homeologous pairing between A and B genomes (Singh and Moss 1982; Wynne and Halward 1989). However, reports of occasional rod or ring multivalents (Husted 1936; Wynne and Halward 1989) in progeny of crosses between different botanical types have been considered to be evidence for segmental allopolyploidy in the cultivated species.

Measurements of fertility of the [*A. batizocoi* × (*A. cardenasii* × *A. diogoi*)]$^{2\times}$ hybrid determined a high degree of sterility, in accord with earlier reports of crosses involving A-genome diploids and *A. batizocoi* (Gibbons and Turley 1967; Smartt *et al.* 1978a). Self-fertility was restored in the amphidiploid, with 89% of pollen stainable, although cross-fertility with *A. hypogaea* was somewhat lower. This behavior of TxAG-6 also fits the definition of genome allopolyploidy. TxAG-6 can be considered as part of the secondary gene pool, GP-2, for *A. hypogaea*, as is *A. monticola*, the only other tetraploid species known in section Arachis (Wynne and Halward 1989).

Phylogenetic relationship of the synthetic amphidiploid to the origin of cultivated peanut: Our marker data (Table 1) agree with recent molecular (Kochert *et al.* 1996) and cytogenetic (Raina and Mukai 1999) studies suggesting that *A. cardenasii*, *A. diogoi*, and *A. batizocoi* were unlikely to be ancestors of *A. hypogaea*. If *A. hypogaea* were derived from a recent hybridization involving *A. cardenasii* and/or *A. batizocoi* with little genomic rearrangement thereafter, it would be expected that a proportion close to the upper limit of 50% of the bands present in *A. hypogaea* would also be present in each *A. cardenasii* and/or *A. batizocoi*. However, *A. cardenasii*, *A. diogoi*, and *A. batizocoi* were much less closely related to *A. hypogaea*, sharing 12–21% of the bands with *A. hypogaea*. Therefore, TxAG-6 represents a synthetic polyploid derived from species that are not in the direct lineage of the cultigen, yet produced progeny with predominantly disomic pairing in crosses with the cultigen. This has been useful for introducing meaningful genetic variability into peanut, with the potential for satisfactory fertility (Burow *et al.* 1996; Choi *et al.* 1999; Simpson and Starr 2001).

These experiments raise questions regarding the classification and nature of the B genome. *A. batizocoi* had been referred to in the literature as a B-genome diploid, and one genome (presumably that derived from *A. batizocoi*) of TxAG-6 pairs preferentially with the *A. hypogaea*

B genome, suggesting similarity. However, other data (Kochert *et al.* 1996; Raina and Mukai 1999) suggest that *A. ipaënsis* is the likely B-genome donor to *A. hypogaea*. Pollen stainability (Krapovickas and Gregory 1994) and cross-compatibility (C. Simpson, unpublished data) data do not support a close relationship between *A. batizocoi* and *A. ipaënsis*. Indeed, Krapovickas and Gregory (1994) consider *A. ipaënsis* to have developed genetic isolation mechanisms equal to those that distinguish *A. duranensis* (A genome) and *A. batizocoi* (putatively B genome). However, the geographic origins of the two species are reasonably proximal (Krapovickas and Gregory 1994), and the apparent ability of *A. batizocoi* to pair effectively with a set of chromosomes likely derived from *A. ipaënsis* suggests that differences may not be as great as thought previously. Some molecular data place *A. batizocoi*, *A. ipaënsis*, and *A. glandulifera* more closely related to each other than to A-genome diploids (Halward *et al.* 1991). It is possible that, as evidenced by their survival as distinct species, *A. batizocoi*, *A. ipaënsis*, and *A. glandulifera* have developed special mechanisms of reproductive isolation; species that fail to do so tend to be assimilated and cease to exist as distinct species (Otte and Endler 1989). Study of an *A. batizocoi* × *A. ipaënsis* hybrid may shed new light on the issue.

Genome rearrangements and speciation: *Rapid rearrangements:* Recent studies have suggested that polyploid speciation triggers rapid genomic restructuring (Song and Osborn 1995; Chen *et al.* 1998; Liu *et al.* 1998). Study of the peanut synthetic polyploid gives evidence for similar processes at work. First, 5% of the mapped TxAG-6 alleles could not be found in any of the five parents or ancestors (*A. hypogaea*, TxAG-6, and the three wild diploids). Such instability has been noted in amphidiploids of Brassica (Song and Osborn 1995) and explained as possible gene conversion or small-scale chromosome rearrangements. In amphidiploid wheat lines, nonrandom elimination of DNA sequences from one of two pairs of homeologous chromosomes was observed (Feldman *et al.* 1997), with evidence for DNA methylation, but not intergenomic recombination (Liu *et al.* 1998). Amphidiploid production in Arabidopsis is associated with rRNA gene silencing by DNA methylation and nucleolar dominance (Chen *et al.* 1998; Furner *et al.* 1998). Polyploidization has been accompanied by activation of transposons in maize (Gaut *et al.* 2000).

Non-Mendelian segregation may reflect either fixation of deleterious alleles or structural or genic divergence associated with speciation. In peanut, a significant number (25%) of markers gave evidence of segregation distortion, and 68% of the markers had an excess of donor (synthetic amphidiploid) alleles. The simplest explanation is fixation of deleterious alleles in the cultigen. In other species, certain chromosomal regions cannot be introduced from unadapted germplasm and maintained stably; for example, in cotton, strong epistatic interactions and selection have been identified (Jiang *et al.* 2000).

Genome affinity: Major rearrangements between homeologous chromosomes in TxAG-6 may contribute to differences in genome affinity. There was evidence for large structural differences in the LG1/LG11 pair and weaker evidence for possible rearrangements in the LG7/LG17, LG4/LG14, and LG5/LG15 pairs. Other homeologous linkage group pairs appeared to be collinear to the degree of resolution afforded by this experiment. This is in agreement with cytological observations (Smartt *et al.* 1978a) suggesting only a limited ability of chromosomes of *A. cardenasii* and *A. batizocoi* to pair; in diploid F_1 hybrids, fewer than six bivalents were observed. This does not explain disomic pairing of collinear homeologs in the tetraploid, but it has been suggested (Smartt and Stalker 1982) that differentiation involves mostly small segments that cannot be detected easily at the current level of resolution. In other species, it has been noted (Stebbins 1950) in crosses involving chromosomes without gross structural differences that differences in genome affinity are nonetheless observed. The *Ph-1* locus in wheat demonstrates that pairing affinity depends not solely on structural differences but on genetic factors as well (Okamoto 1957; Sears 1976).

Evidence for ancient duplication in peanut: The existence of three collinear markers in two linkage groups (LG5 and LG13) and the weaker relation between their homeologs (LG15 and LG3) may suggest ancient duplication of part of that linkage group pair, resulting from a duplication predating the divergence of the A and B genomes of peanut. The existence of additional comapping pairs of markers suggests the possibility of additional duplication, but further evidence is needed to draw conclusions. Evidence for ancient duplication in relatively recent polyploids has been obtained in Brassica, in which there is evidence for duplication of the genome subsequent to polyploid formation from ancestral species currently considered to be diploid (Kowalski *et al.* 1994; Paterson *et al.* 1996, 2000). In legumes, basic chromosome numbers are 11 for Phaseolus and Vigna and 10 for Glycine (assuming an ancient polyploidization event) and Arachis. No consensus legume chromosome number of 5 has been proposed to date, although Medicago's basic chromosome number of 6 could be close to an ancestral chromosome complement.

Comparative genetics of peanut and other legumes: The map of tetraploid peanut fills an important void in legume biology, as peanut was the last major cultivated legume lacking a map of all chromosomes. Work in progress to merge the tetraploid and diploid (Halward *et al.* 1993; Garcia *et al.* 1995) maps will unify peanut genetics. By mapping Phaseolus, Vigna, Glycine, and Medicago probes, it may be possible to integrate peanut into a prototypical legume map (Boutin *et al.* 1995). Such a project will be facilitated by development of

comprehensive physical maps of Glycine and Medicago and physical mapping of a significant number of sequenced expressed sequence tags. Development of peanut genomic tools is also essential for this and other genetic analysis; synthesis of a peanut bacterial artificial chromosome library is key to this goal. An enhanced-density tetraploid map can serve as the basis for integration of genetic and future physical maps.

Future of introgression into A. hypogaea: Continued introduction of variability into peanut is important both for understanding the Arachis genome and in reducing genetic vulnerability of the cultigen. Many useful alleles have been identified in the A-genome diploid species examined (Stalker and Moss 1987; Singh and Simpson 1994). Future amphidiploid formation can be simplified by use of one A-genome diploid parent, as intercrossing two A-genome diploids does not confer special advantages because one-half the complement of the AA′ hybrid is lost when crossed to a B-genome diploid. Identification and utilization of other potential B-genome donors in addition to *A. batizocoi* (17 accessions) and *A. ipaënsis* (2 accessions) would be useful for expansion of the gene pool. In a similar manner, recreation of the hypothesized [*A. duranensis* × *A. ipaënsis*]$^{4\times}$ cross and study of *A. batizocoi* and *A. ipaënsis* would benefit the understanding of the origin of cultivated peanut. In addition to generation of new amphidiploids, continued introgression using the existing cross will maximize utilization of germplasm. Analysis of quantitative traits by the advanced-backcross-quantitative trait locus approach (Tanksley and Nelson 1996) and generation of near-isogenic introgression lines (Eshed and Zamir 1995) should facilitate the identification and utilization of valuable genes from exotic peanut species and also basic understanding of legume comparative genetics.

The authors thank Renée Baggett, Julie Dunlap, Mancha C. M. Reddy, Elizabeth Tomaszewski, Greg Warr, and Gunawati Gunawan for technical assistance, and Drs. Peter Morrell and Peng Chee for reviewing the manuscript. Aspects of this work were supported by funds from the Texas Agricultural Experiment Station Research Enhancement Project, granted to J.L.S., C.E.S., and A.H.P.; by United States Department of Agriculture (USDA)-National Research Initiative Competitive Grants Program (NRICGP) grants 95-37302-2150 to J.L.S., M.D.B., A.H.P., and C.E.S.; by USDA-NRICGP grant 97-35300-4584 to C.E.S., A.H.P., and J.L.S.; by USDA-NRICGP grant 98-35302-6879 to J.L.S., C.E.S., A.H.P., and M.D.B.; and by the Texas Advanced Technology Program grant 999902-094 to C.E.S., J.L.S., A.H.P., and M.D.B. Probe requests should be directed to Dr. Paterson at the University of Georgia.

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Communicating editor: T. F. C. Mackay