Published February 14, 2019

RESEARCH

Introgression Analysis and Morphological Characterization of an *Arachis hypogaea* × *A. diogoi* Interspecific Hybrid Derived Population

Wesley G. Hancock, Shyam P. Tallury, Thomas G. Isleib, Ye Chu, Peggy Ozias-Akins, and H. Thomas Stalker*

ABSTRACT

Cultivated peanut (*Arachis hypogaea* L.) is an economically important crop grown around the world. Compared with the entire *Arachis* genus, cultivated peanut germplasm has low levels of genetic diversity for several economically important traits, resulting in the need for alternative sources of favorable alleles. Wild diploid species of *Arachis* are a source of such alleles to improve cultivated peanut for many economically important traits. An *A. hypogaea* × *A. diogoi* Hoehne introgression population was produced via the triploid–hexaploid method; the fourth generation after tetraploidy was used to initiate this study. The introgression lines were genotyped using a single nucleotide polymorphism (SNP) marker array to estimate the percentage of *A. diogoi* chromatin introgression. Morphologically, the introgression lines varied for an array of measured traits, with the majority being intermediate to the two parents. The average amount of *A. diogoi* genome introgressed was 8.12% across the tetraploid genome and ranged from 3.00 to 18.14% on individual chromosomes. The average *A. diogoi* introgression across all lines was 7.70% and ranged from 0.17 to 51.12%. Principal component analysis of morphological data and SNP markers revealed similarities and groupings of introgression lines. This introgression population demonstrates the potential of using wild diploid *Arachis* species for peanut improvement and has great potential for use in cultivated peanut breeding programs.

W.G. Hancock, T.G. Isleib, and H.T. Stalker, Dep. of Crop and Soil Sciences, North Carolina State Univ., Raleigh, NC 27695; S.P. Tallury, Plant Germplasm Resources Conservation Unit, USDA-ARS, Griffin, GA 30223; Y. Chu and P. Ozias-Akins, Dep. of Horticulture, Institute of Plant Breeding, Genetics and Genomics, Univ. of Georgia, Tifton, GA 31793. Received 24 July 2018. Accepted 18 Nov. 2018. *Corresponding author (tom_stalker@ncsu.edu). Assigned to Associate Editor Hussein Abdel-Haleem.

Abbreviations: LD, linkage disequilibrium; NCDA&CS, North Carolina Department of Agriculture and Consumer Services; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism.

Peanut (*Arachis hypogaea* L.) is an allotetraploid ($2n = 4x = 40$) crop species that arose from hybridization between two diploid ($2n = 2x = 20$) progenitors followed by chromosome doubling (Kochert et al., 1996; Stalker, 1997; Seijo et al., 2007). Seijo et al. (2004, 2007), along with Ramos et al. (2006), provided fluorescent in situ hybridization (FISH) and genomic in situ hybridization (GISH) evidence, and more recently, Bertioli et al. (2016) provided genome sequence information supporting *A. duranensis* Krapov. & W.C. Gregory and *A. ipaënsis* Krapov. & W.C. Gregory as the progenitor species of *A. hypogaea*. Cultivated peanut has two subgenomes, with ancestors of *A. duranensis* being the female parent and donor of the "A" genome and *A. ipaënsis* being the donor of the "B" genome (Kochert et al., 1996; Seijo et al., 2004, 2007; Moretzsohn et al., 2013; Bertioli et al., 2016). Genome duplication produced a new polyploid species that was isolated reproductively from its diploid progenitors and relatives, and thus limited genetic introgression from the diploid relatives has occurred, resulting in the relatively narrow genetic base of cultivated peanut (Halward et al., 1991, 1992). Therefore, additional sources of favorable

Published in Crop Sci. 59:640–649 (2019).
doi: 10.2135/cropsci2018.07.0461

genetic variation influencing economically important traits are needed for crop improvement.

The genus *Arachis* is composed of 77 naturally occurring diploid, three aneuploid, and three tetraploid species, many of which could serve as sources of valuable alleles for the improvement of economically important traits in cultivated peanut (Stalker and Moss, 1987; Valls and Simpson, 2005; Krapovickas and Gregory, 2007; Valls et al., 2013; Santana and Valls, 2015; Valls and Simpson, 2017; Stalker, 2017). Technical challenges exist in using wild *Arachis* germplasm for improving cultivated peanut, mainly due to sterility caused by ploidy and genomic differences, hybrid necrosis, and cross-incompatibilities (Stalker and Moss, 1987). Several methods have been used to introgress favorable wild diploid species genes into cultivated peanut (Stalker et al., 1979; Stalker and Wynne, 1979; Stalker and Moss, 1987; Simpson, 1991, 2001). All strategies have involved the use of chromosome doubling agents to produce progenies with much greater levels of fertility.

One method, referred to as the "triploid–hexaploid introgression method," involves hybridizing *A. hypogaea* with a diploid wild species and chromosome doubling the sterile triploid ($2n = 3x = 30$) with colchicine to restore fertility at the hexaploid ($2n = 6x = 60$) level, followed by self-pollination over many generations during which spontaneous chromosome loss occurs, resulting in 40-chromosome interspecific hybrid derivatives. The goal of self-pollination at a high ploidy level is to increase frequency of recombination between the wild and cultivated species. Little is known about how spontaneous chromosome loss occurs, but typically it is random and infrequent; therefore, the method requires many generations of self-pollination before tetraploids appear (Stalker et al., 1979; Stalker, 2017).

Successful use of the triploid–hexaploid introgression method was demonstrated in crosses between the diploid species *A. cardenasii* Krapov. & W.C. Gregory and *A. hypogaea* (Smartt and Gregory, 1967; Stalker et al., 1979). Many *A. cardenasii*-derived materials possess high levels of disease and insect resistance (Stalker, 2017), and numerous germplasm lines and cultivars derived from *A. cardenasii* × *A. hypogaea* have been released (Stalker and Beute, 1993; Stalker et al., 2002a, 2002b; Stalker and Lynch, 2002; Isleib et al., 2006; Tallury et al., 2013; Stalker, 2017). Molecular marker analysis of *A. hypogaea* × *A. cardenasii*-derived interspecific hybrids have shown that introgression occurs into both *A. hypogaea* subgenomes (Garcia et al., 1995). In addition, it has been proposed that the mechanism of wild species chromatin introgression from *A. cardenasii* into the *A. hypogaea* genome is chromosome recombination rather than chromosome substitution (Garcia et al., 1995). These introgressed chromosome segments also appear to be in large blocks (Garcia et al., 1995; Nagy et al., 2010).

Much of the multiple disease resistance present in some breeding programs and peanut cultivars can be attributed to the abovementioned *A. cardenasii*-derived introgression lines (Stalker, 2017). However, wide use of the same disease resistance mechanisms may place great selection pressure on pathogens, resulting in a decrease in the effectiveness of the resistance or rendering it ineffective over time (McDonald and Linde, 2002). Thus, additional sources of multiple disease resistance in peanut are needed. One wild diploid species, *A. diogoi* Hoehne (syn. *A. chacoense* nom. nud.), specifically accession GKP 10602 (PI 276235), is highly resistant to many diseases affecting peanut production (Abdou et al., 1974; Company et al., 1982; Subrahmanyam et al., 1985; Lyerly et al., 2002; Stalker, 2017). The objectives of this research were to characterize an *A. hypogaea* × *A. diogoi* introgression population developed by the triploid–hexaploid method. Molecular markers were used to determine whether tetraploid introgression lines have *A. diogoi* chromosome substitutions or whether recombination occurred between the diploid and tetraploid parents. The percentage of *A. diogoi* chromatin that was introgressed into each line was determined, as well as the amount of introgression present across the *A. hypogaea* genome. Morphological characteristics of the introgression lines also were evaluated.

MATERIALS AND METHODS

Introgression Population Development

During the summer of 2000, the large-seeded Virginia-type peanut cultivar 'Gregory' (Isleib et al., 1999) was hybridized with *A. diogoi* accession GKP 10602 (PI 276235) to produce sterile triploid interspecific F_1 hybrids ($2n = 3x = 30$). In 2001, cuttings from one sterile triploid were treated with a 0.2% colchicine solution for 8 h at room temperature to double the chromosome number and restore fertility at the hexaploid ($2n = 6x = 60$) chromosome level. Cuttings were treated with Rootone rooting hormone (GardenTech) and placed in sand in a mist chamber under shade for ~6 wk to develop roots. The plants were grown to maturity in the greenhouse and one seed was recovered, which was a hexaploid individual. The seed was planted in a 38-cm by 52-cm flat containing soil, and the plant was cytologically confirmed as being hexaploid ($2n = 6x = 60$). It was grown to maturity and seed were harvested by hand. The resulting progeny from this hexaploid individual were then spaced planted and self-pollinated with no artificial selection for 11 generations (2002–2012) at the North Carolina Department of Agriculture and Consumer Services (NCDA&CS) Sandhills Research Station near Jackson Springs in Moore County, North Carolina. Plots were grown according to common agricultural practices for peanut production in North Carolina (North Carolina Cooperative Extension, 2017) with the exception of planting and harvesting methodologies. For planting, seeds were sown in 5.5-cm by 5.5-cm peat pots (Jiffy-pots, Jiffy International) containing equal parts steamed sand, steamed topsoil, and Fafard 2P soil mix in the greenhouse and transplanted to the field in mid-May. Plants were hand dug and pods were manually removed from each plant in mid-October.

During 2011, one of the field-grown hexaploid plants produced three progenies that had an *A. hypogaea* plant growth

habit when grown in 2012. Flow cytometric analysis confirmed that these progenies were tetraploid (Tallury et al., 2014). In 2013, 12 individual tetraploid plants were selected on the basis of seed size (both large and small seeded lines), and in 2014, 87 introgression lines were derived from these selections. The 87 introgression lines were advanced using small plots of bulked seed for three generations. During the fall of 2016, one seed from each of the 87 introgression lines was planted in a separate pot in the greenhouse, grown to maturity, and seed harvested by hand. During the summer of 2017, seed of each single plant progeny from each of the 87 introgression lines were planted in a seed increase nursery at the NCDA&CS Peanut Belt Research Station near Lewiston-Woodville in Bertie County, North Carolina. For this nursery, the seeds of each introgression line were planted in two-row plots 3.7 m in length with 25-cm within-row seed spacing. Plots were grown according to common agricultural practices for peanut production in North Carolina (North Carolina Cooperative Extension, 2017), and seeds from each introgression line were harvested in bulk.

Phenotyping of Morphological Characteristics

Introgression lines were evaluated for 13 morphological characteristics on plants in the seed increase nursery and on pods and seeds harvested from both the 2016 and 2017 seed increase nurseries at the Peanut Belt Research Station. The following morphological traits were evaluated: pod length (cm), pod width (cm), seed length (mm), seed width (mm), number of seeds per pod, seed weight (g), meat content (%), growth habit (spreading = 1, semibunch = 2, bunch = 3, segregating bunch and spreading = 4, prostrate = 5), presence of flowers on the main stem, flowering pattern on lateral stems (alternate pattern of reproductive nodes = 1, sequential pattern of reproductive nodes = 2), prominence of the main stem (not prominent = 1, semiprominent = 2, prominent = 3), plant height (cm), and canopy width (cm). Using these data, three other traits were calculated: pod length/width ratio, seed length/width ratio, and percentage row closure. Row closure was estimated as the ratio of canopy width to row spacing (91.4 cm). Pod and seed measurements were taken on 20 random pods from each line. Seed weight was evaluated on seed lots from one to two replications in 2016 and one replication in 2017. Meat content was estimated by the ratio of seed weight to pod weight of 50 randomly selected pods. Growth habit and visibility of the main stem were evaluated on whole plots. Four mature plants were sampled from each plot for branching pattern, plant height, and plant width. The morphological characteristics, percentage row closure, and presence of flowers on the main stem were not included in subsequent analysis resulting in 14 total traits. Principal component analysis (PROC PRINCOMP) of SAS version 9.4 (SAS Institute, 2013b) was performed on the averages for 14 morphological traits. The introgression lines were plotted on two dimensions using the first two principal components.

Molecular Marker Genotyping and Introgression Analysis

One folded leaf consisting of all four leaflets was collected from a single plant from each introgression line and the two parents during the winter of 2016–2017. DNA was isolated using a Qiagen DNAeasy Plant Mini Kit. DNA was quantified using PicoGreen (Thermo Fisher Scientific) and diluted to 30 ng μL^{-1} according to Affymetrix guidelines. Samples were genotyped using the Affymetrix Axiom_Arachis2 48K single nucleotide polymorphism (SNP) array (Clevenger et al., 2017). Quality control and SNP genotype determination were performed using Axiom Analysis Suite version 3.0.1.4 (Thermo Fisher Scientific, 2016) using the default polyploidy threshold parameters. In addition to the 87 introgression lines and the two parents, nine other diploid *Arachis* species were included in the analysis to assist in genotype clustering by Axiom Analysis Suite software and for additional downstream introgression analysis. The nine diploid *Arachis* species included *A. batizocoi* Krapov. & W.C. Gregory accession K 9484; *A. cardenasii* accession GKP 10017; *A. correntina* (Burkart) Krapov. & W.C. Gregory accession GKP 9548; *A. duranensis* accession SeSn 2848; *A. gregoryi* C.E. Simpson, Krapov., & Valls accession V 6389; *A. ipaënsis* accession KGBSPSc 30076; *A. magna* Krapov., W.C. Gregory, & C.E. Simpson accession KG 30097; *A. stenosperma* Krapov. & W.C. Gregory accession V 10309; and *A. villosa* Benth. accession V 12812. All Axiom Analysis Suite array calls were visually checked for each potential SNP marker according to Affymetrix guidelines and manually corrected when the default clustering was not accurate.

The SNP genotypes were converted to a proportional scoring scale, with "0" equal to the SNP marker identifying a homozygous genotype for Gregory, "1" equal to the SNP marker identifying a heterozygous genotype, and "2" equal to SNP marker identifying a homozygous genotype for *A. diogoi* to estimate the total amount of *A. diogoi* introgression. This was estimated using R (R Core Team, 2017) and RStudio version 1.0.143 (RStudio Team, 2017). The SNPs were assigned to subgenome A or B based on the assignments on the SNP chip used for this analysis as assigned by J. Clevenger (personal communication, 2017) and P. Ozias-Akins. Graphical representation of introgression across all chromosomes were prepared using the software GGT2 (van Berloo, 2008). The marker-derived genomic relationship matrix was used for principal component analysis in JMP Genomics 8 (SAS Institute, 2013a) to estimate the genetic relationships among introgression lines.

A preliminary marker–trait association analysis for the 14 morphological traits was conducted to gain insight on the phenotypic contributions of the various introgressed *A. diogoi* alleles in the Gregory genetic background. The introgression line IL 01 was dropped from the analysis as an outlier with respect to the amount of introgression present in that line because it had a very large number of SNPs not present in the other lines and its inclusion would have resulted in a skewed representation of the introgression in the population. Markers detecting no introgression within the population after removal of introgression line IL 01 were dropped from the analysis. Thus, a total of 4300 SNP markers were analyzed to estimate the amount of *A. diogoi* introgression. Markers were eliminated based on linkage disequilibrium (LD, $r^2 > 0.8$) using the LD tagSNP package in JMP Genomics 8 to reduce the total number of markers used in marker–trait association analysis. The LD tagSNP package eliminates redundant information in the marker data set and keeps SNP markers that provide the maximum information (Carlson et al., 2004). The marker–trait association analysis was

conducted in JMP Genomics 8 using the single marker–trait association package. For the marker–trait association analysis, the critical significance level for declaring a significant marker–trait association was set at 0.0001 (logarithm of odds = 3) for all morphological traits.

RESULTS

Morphological Characteristics

Morphologically, the introgression lines varied for all traits including canopy characteristics, plant height, growth habit, and pod and seed traits (Supplemental Table S1). The majority of introgression lines were morphologically intermediate between the two parents. A smaller number of lines were found to be identical to either parent for certain traits. A range of variation among the introgression lines was observed for the measured pod and seed characters (Fig. 1, Supplemental Fig. S1). The overall average for individual seed weight of the 87 introgression lines (0.65 g) was greater than the mid-parent average (0.52 g), but less than the average of Gregory (0.999 g) (Fig. 2). Seed weights for the introgression lines ranged from 0.33 to 1.02 g. A few lines were identified as having seed size equal to or greater than Gregory. For meat content, Gregory (68%) was slightly lower than *A. diogoi* (73%), with the meat content of the introgression lines ranging from 48 to 77% with an average of 67% (Supplemental Table S1). On average, the majority of lines had two seeded pods typical of Virginia-type peanut cultivars, and a small number of lines had one-seeded pods similar to the wild species.

Main stem height and plant width varied across the introgression lines, with the majority of lines being intermediate to the two parents for plant height and slightly closer to *A. diogoi* for plant width. Row closure was estimated from plant width to provide an estimation of canopy closure, an important agronomic trait. At the time of measurement (31 Aug. 2017, 105 d after planting), row closure ranged from 48% for the bunch type lines to 100% for the spreading type lines. Two introgression lines had a bunch growth habit, even though this trait was not observed in either parent. The majority of introgression lines had the alternate branching pattern typical of *A. hypogaea* subsp. *hypogaea*. Although the visual prominence of the main stem was somewhat related to growth habit, variation was observed for this characteristic regardless of growth habit.

The first five principal components accounted for 83% of the total variation, with the first and second principal components accounting for 37 and 20% of the variation, respectively (data not shown). Based on the loadings in the eigenvectors, the seed and pod morphological variables (pod length, pod width, seed length, seed width, and seed weight) had the greatest effects on the first principal component, whereas the plant architectural morphological variables (plant width, growth habit, branching pattern, and main stem prominence) had the greatest effect on the second principal component. Based on the principal components, most introgression lines were more morphologically similar to the cultivated parent than the diploid wild species, with the wild species having an effect on the introgression lines where the two groups are clustered between both parents.

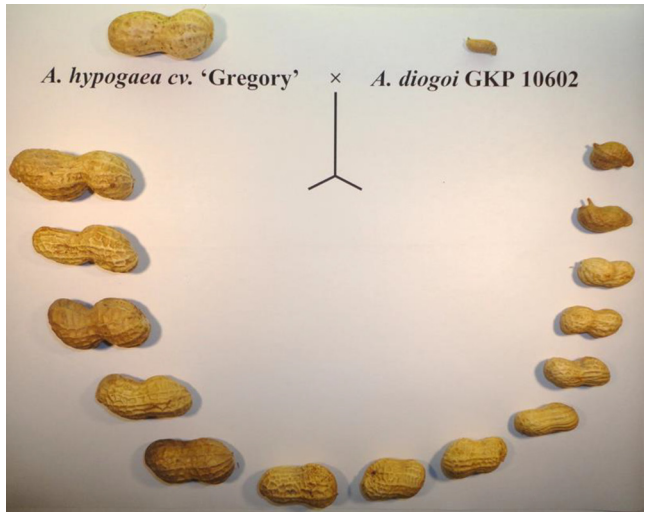

Fig. 1. Variation in mature peanut pod shape and size of select *A. hypogaea* × *A. diogoi* introgression lines, and the parents Gregory and *A. diogoi*, grown at the Peanut Belt Research Station, Lewiston-Woodville, NC, in 2016.

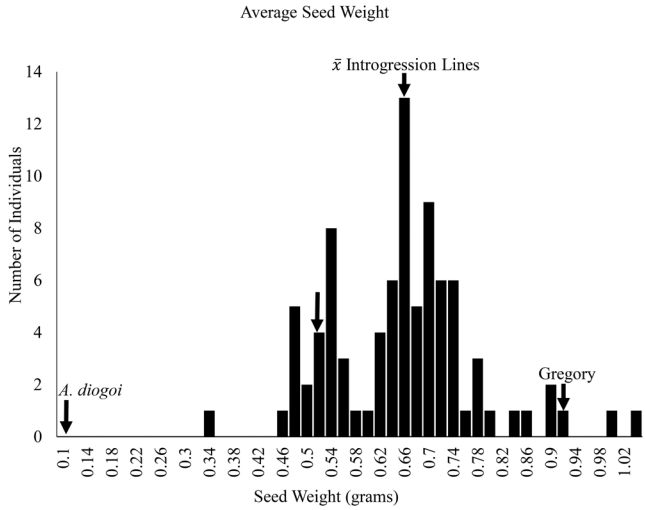

Fig. 2. Distribution of seed weight for 87 *A. hypogaea* × *A. diogoi* introgression lines. Means for introgression lines, the two parents *A. diogoi* and Gregory, and the mid-parent average are indicated by arrows.

Marker Polymorphism

A diverse set of unique SNP markers was also found to differentiate *A. hypogaea* from other diploid *Arachis* species. The final SNP marker set consisted of a total of 7017 markers, all of which were polymorphic between the two parental lines *A. diogoi* and Gregory. The 7017 markers densely covered the genome of *A. hypogaea* with an average

of 351 markers per chromosome. Of these 7017 markers, 6070 were polymorphic between Gregory and *A. batizocoi*, 6289 were polymorphic between Gregory and *A. cardenasii*, 6212 were polymorphic between Gregory and *A. correntina*, 5771 were polymorphic between Gregory and *A. duranensis*, 6524 were polymorphic between Gregory and *A. gregoryi*, 5833 were polymorphic between Gregory and *A. ipaënsis*, 5508 were polymorphic between Gregory and *A. magna*, 6529 were polymorphic between Gregory and *A. stenosperma*, and 6162 were polymorphic between Gregory and *A. villosa*.

Introgression Analysis

Of the 7017 total markers polymorphic between Gregory and *A. diogoi*, 391 did not identify *A. diogoi* introgression in any of the introgression lines, whereas 6626 markers exhibited introgression of *A. diogoi* in one or more lines (Table 1). These markers showed two different *A. diogoi* chromatin introgression patterns: (i) substitution of *A. diogoi* chromatin into either subgenome of *A. hypogaea*, or (ii) small and large insertions of *A. diogoi* chromatin. Across all chromosomes, the average amount of *A. diogoi* introgression was 8.12% and ranged from 3.00 to 18.14%. There was a greater amount of introgression on chromosomes A.04/B.04, A.05/B.05, and A.06/B.06 and the least amount on chromosome A.07/B.07

A subset (1205 SNP markers) of the total number of SNP markers was found to differentiate the two *A. hypogaea* subgenomes based on SNP marker polymorphisms between the progenitor species, and these markers were used to estimate the amount of *A. diogoi* chromatin introgression due to the substitution introgression pattern. These markers showed that *A. diogoi* chromatin was introgressed into both the *A. duranensis* (A) and the *A. ipaënsis* (B) subgenomes of *A. hypogaea*. However, percentages were not calculated because of the tentative assignments of SNPs in the two genomes, since there has not been genetic confirmation of assignments. In all cases, however, the *A. diogoi* allele replaced the progenitor allele. Tetrasomic recombination was identified on chromosomes A.04, A.06, B.04, and B.06 with 129 total markers showing tetrasomic recombination on the four chromosomes of *A. hypogaea*. The *A. diogoi* allele was present in zero to four copies for the 129 markers showing tetrasomic recombination.

Varying levels of introgression were observed across the 87 introgression lines. The average introgression for all lines was 7.70% and ranged from 0.17 to 51.12% (Supplemental Table S2). Two introgression lines were outliers with respect to the amount of introgression present, with line IL 36 having the least amount of introgression (0.17%) and line IL 01 having the most introgression present (51.12%), respectively. Excluding these two lines, the majority of individuals (85 of 87 lines) had an average introgression of 7.28% and ranged from 4.49 to 15.89%. No introgression line harbored all of the introgressed *A. diogoi* SNP markers analyzed. *Arachis diogoi* introgressions were present in large blocks across numerous neighboring markers and in other cases only in single markers (shown as graphical genotypes in Supplemental Fig. S2). Similar patterns of introgression were observed across several introgression lines. These similar introgressions across numerous introgression lines may have been a product of a single introgression event that occurred in earlier generations of population development in the hexaploid materials.

A principal component analysis on the molecular marker data was performed to provide an estimate of

Table 1. Genome-wide estimation of percentage *A. diogoi* introgression per chromosome and totals for markers for the 87 *A. hypogaea* × *A. diogoi* introgression lines.

Chr.†	Total no. of SNP‡ markers per chromosome	No. of SNP markers detecting *A. diogoi* introgression§	Avg. *A. diogoi* introgression¶
			%
A.01/B.01	690	654	5.25
A.02/B.02	759	726	5.16
A.03/B.03	760	724	8.11
A.04/B.04	591	552	12.75
A.05/B.05	630	599	16.98
A.06/B.06	659	634	13.96
A.07/B.07	971	937	3.05
A.08/B.08	567	521	5.99
A.09/B.09	836	771	4.32
A.10/B.10	564	518	5.69
Total	7017	6626	–
Avg.	665	–	8.12

† Chr., chromosome. Chromosome information supplied by Affymetrix.

‡ SNP, single nucleotide polymorphism.

§ SNP markers identifying introgression of *A. diogoi* in one or more lines.

¶ Average *A. diogoi* introgression (sum of the number of lines harboring an *A. diogoi* genotype at each SNP marker across all markers of the corresponding chromosome divided by total number of marker calls of the corresponding chromosome).

genetic relatedness among the introgression lines. The first two principal components accounted for 77.4% of the total variation, with the first and second principal component accounting for 69.5 and 7.9% of the variation, respectively (Fig. 3). The two-dimensional separation of the lines in a scatterplot of the first two principal components shows that many lines are highly similar genetically. Four distinct groups can be identified from this separation with the majority of individuals clustering around both parents. Thirty-four introgression lines clustered tightly together, and the genetic relationship among these 34 introgression lines, estimated as the correlation between SNP markers, ranged from $r^2 = 0.70$ to $r^2 = 0.99$. Two smaller groupings of four and seven introgression lines were observed. The clusters did not correspond with the three original tetraploid revertants, as progeny from each of these three were present in each of the clusters. Thus, it does not appear as though introgression occurred during the first hexaploid generation, but more likely occurred over many of the 12 generations of selfing during subsequent years.

The preliminary marker–trait association analysis revealed 1082 total marker–trait associations with the 14 morphological traits (Supplemental Table S3). Chromosomes A.08 (23) and B.06 (112) harbored the lowest and highest number of significant ($P < 0.0001$) markers, respectively. Individual SNP markers were associated with one to nine morphological traits resulting in 286 unique SNP markers with significant ($P < 0.0001$) marker–trait associations. However, the population was highly variable and many of the traits are very complex, and the traits with large numbers of marker associations do not present usable data for selection. No marker–trait associations were identified for seed width. Two, three, and five significant ($P < 0.0001$) marker–trait associations were identified for branching pattern, pod ratio, and number of seeds per pod, respectively.

DISCUSSION

A wide range of values for the morphological characteristics collected on introgression lines was observed, presumably due to alien *A. diogoi* chromatin introgressed into the Gregory genetic background. Pod and seed size are extremely important for incorporation of exotic germplasm in Virginia market-type breeding programs due to the stringent requirements for Virginia-type cultivars. Thus, selection on the basis of large seed size was conducted among the progeny of the tetraploid plants derived from self-pollinating the hexaploid individual. A number of introgression lines were identified that had acceptable seed size for a Virginia market-type cultivar. The majority of lines possessed seed size acceptable for use in a runner market-type breeding program. Although the seed size was acceptable for a number of lines, no data were recorded on market grades or yield in the present study.

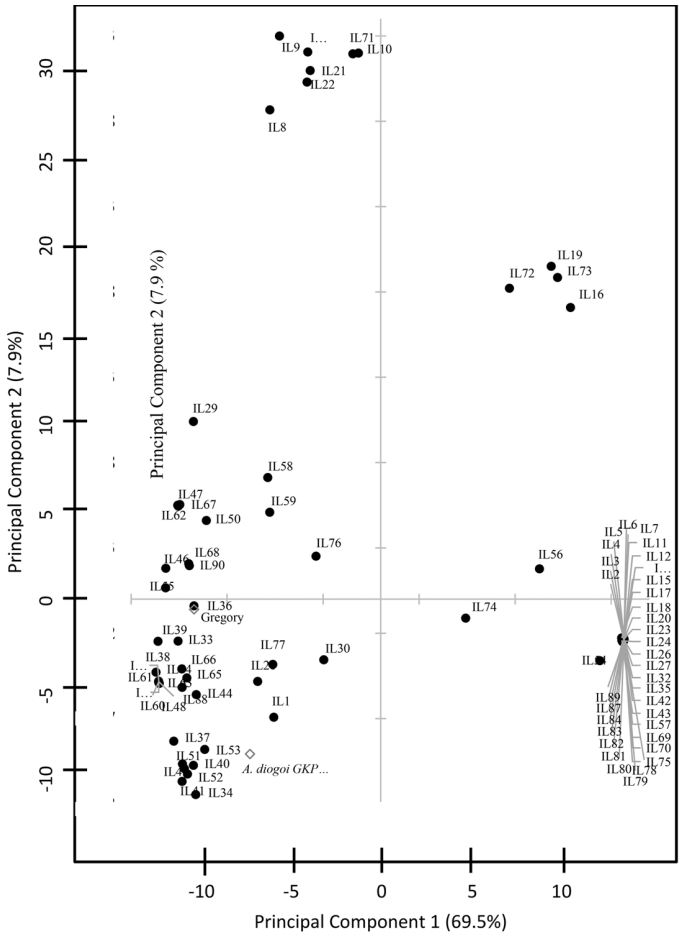

Fig. 3. Visualization of genetic relationships between *A. hypogaea* × *A. diogoi* introgression lines through the projection of introgression lines and parents onto the first and second principal components (PC1 on the horizontal axis and PC2 on the vertical axis) from principal component analysis of single nucleotide polymorphism (SNP) marker data.

Previous studies indicated that 40-chromosome derivatives derived from the triploid–hexaploid interspecific hybridization between *A. cardenasii* and *A. hypogaea* had large blocks of introgression, were intermediate to both parents morphologically, and possessed high levels of resistance to numerous plant pathogens that were derived from *A. cardenasii* (Stalker et al., 1979; Stalker, 1984; Garcia et al., 1995, 1996). Likewise, large blocks of *A. diogoi* introgressed chromosome segments were observed in addition to smaller regions of single markers that detected introgression. The findings in this study were similar to those of Garcia et al. (1995).

A reduction in recombination has been found in other introgression populations (Garcia et al., 1996; Nagy et al., 2010) and may be expected in the *A. diogoi* lines. Nagy et al. (2010) found recombination to be suppressed around the root-knot nematode resistance gene (*Rma*) harbored on an alien chromosome segment that comprised one-third to one-half of chromosome A.09. The suppression of recombination around introgressed regions is also found

in tomato (*Lycopersicon esculentum* Miller) introgression populations (Chetelat et al., 2000; Canady et al., 2006).

Similar to Garcia et al. (1995, 1996) for *A. cardenasii* introgression lines, our results indicate that the mechanism of *A. diogoi* chromatin introgressions is not due to chromosome substitution. Rather, it appears that introgression is due to crossing-over and reciprocal recombination involving both subgenomes. This is evident because in no case was an entire *A. diogoi* chromosome or chromosome arm found in an introgression line. Bivalent chromosome paring in *A. hypogaea* is most common, although multivalent chromosome paring has been reported (Husted, 1936; Stalker and Wynne, 1979; Stalker, 1985; Leal-Bertioli et al., 2015). Cytological analysis of triploid individuals derived from the interspecific hybridization of *A. hypogaea* with diploid *Arachis* species shows that 10 bivalents and 10 univalents are typically observed, but trivalents have also been reported (Company et al., 1982; Stalker, 1985). The presence of intergenomic pairing (Singh and Moss, 1984) and the presence of trivalent formation within triploid hybrids (Company et al., 1982), although somewhat rare, are likely the basis of the genetic exchange and recombination between chromosomes of the wild species and *A. hypogaea*, which are manifested over the numerous cycles of self-pollination at a high ploidy level that is necessary to develop such individuals.

The mechanism of chromosome elimination in the triploid–hexaploid method of producing tetraploid introgression lines in *Arachis* is not fully understood. The unique genomic composition of two different parental genomes within one nucleus can lead to intergenomic conflicts such as chromosome elimination. Chromosome elimination has been reported in interspecific hybrids within other crop species genera such as *Hordeum* (Subrahmanyam and Kasha, 1973) and *Nicotiana* (Hancock et al., 2015). Hypotheses to explain the mechanisms of chromosome elimination after interspecific hybridization in various crop species include differences in timing of the mitotic cycle between the two species, histone changes resulting in the failure of centromeres to attach to the spindle microtubules, host-specific nucleases capable of degrading alien chromatin, or the formation of micronuclei followed by selective removal (Kasha and Kao, 1970; Subrahmanyam and Kasha, 1973; Finch, 1983; Gernand et al., 2005; Ravi and Chan, 2010, 2013). In these previous studies, chromosome elimination typically occurs over one generation, resulting in haploid individuals. It is unclear if any of these chromosome elimination mechanisms are at play in the triploid–hexaploid method of *Arachis* hybridization, primarily due to the fact that hexaploid individuals are maintained at that ploidy level for a number of generations, during which time the selective elimination of chromosomes occurs randomly but presumably in a few generations. Although requiring a large time and labor expense, cytological investigations during the numerous generations of inbreeding of hexaploid individuals would be needed to confirm the mechanism and rate of chromosome elimination.

A greater amount of *A. diogoi* introgression into the A genome of cultivated peanut is expected, since *A. diogoi* is classified as an A-genome species. Chromosomal homeolog assignment of the SNPs in this study is tentative in the absence of genetic data given the demonstration of homeologous chromosome pairing and recombination. However, evidence suggests that introgression into both subgenomes has occurred.

Garcia et al. (1995) conducted a restriction fragment length polymorphism (RFLP) evaluation of an *A. hypogaea* × *A. cardenasii* population derived via the triploid–hexaploid introgression method and observed most introgression into the A genome, but also introgression into the B genome. Thus, the results in this study were not unexpected. Because there is a high degree of chromosome synteny reported between *A. duranensis* and *A. ipaënsis* and the corresponding A and B subgenomes of *A. hypogaea*, in addition to a high degree of synteny (∼99%) between the two subgenomes within *A. hypogaea* (Seijo et al. 2004, 2007; Foncéka et al., 2009; Guo et al., 2012; Bertioli et al., 2009, 2016), there is likely a considerable amount of chromosome pairing between the two genomes. This sequence synteny likely results in pairing between chromosomes of either genome and the *A. diogoi* chromosomes within the hexaploid lines, followed by the potential for recombination and introgression of *A. diogoi* chromatin into either *A. hypogaea* subgenome. The *A. diogoi* introgression into the B genome could be biased upward due to ambiguities associated with preliminary chromosome assignments of the Affymetrix Axiom_Arachis2 SNP array caused by the high degree of similarity between subgenomes. Although there may be a bias of having assignments to the B genome, the critical point of the study is to illustrate *A. diogoi* introgression to *A. hypogaea* and its potential for cultivar improvement.

Marker–trait association analysis of morphological traits revealed a large number of markers significantly associated with certain traits in addition to a large number (226) of markers significantly associated with two or more traits. Future studies are needed to reduce the number and verify the associated markers with these traits of interest. Much of the *A. diogoi* introgression harbors unfavorable genetic variation influencing important morphological traits, such as smaller seed size, and molecular markers linked to traits of interest can be used in marker-assisted selection schemes. Nevertheless, with the array of *A. diogoi* introgression present in the population and its effect on plant morphological traits, all introgression lines are of the Virginia type with regards to plant structural classification.

Here we report the successful development of an interspecific hybrid derived population through the use

of the triploid–hexaploid introgression method with two *Arachis* species. This population will be phenotyped for an array of disease reactions to facilitate the determination of marker–trait associations that could be used in marker-assisted selection schemes. Additional crosses will be needed to move favorable introgressions into acceptable genetic backgrounds, and attempts to break any linkages between undesirable and desirable genetic variation should be made more efficient through the application of marker-assisted selection. Additionally, this research demonstrates the value of SNP marker arrays to peanut research and the genetic improvement of cultivated peanut.

This *A. diogoi* interspecific derived introgression population is highly variable even after selection for *A. hypogaea* morphological traits, and all progenies are subsp. *hypogaea*, var. *hypogaea*, an important consideration for use in most US peanut breeding programs. An array of *A. diogoi* introgression was observed on all chromosomes, with the majority of introgression on chromosomes A.04/B.04, A.05/B.05, and A.06/B.06 of both genomes. A significant amount of introgression into both the A and B genomes was observed even though *A. diogoi* is an A genome species.

Conflict of Interest

The authors declare that there is no conflict of interest.

Supplemental Material Available

Supplemental materials for this article are available online.

Acknowledgments

This research was supported in part by the National Science Foundation Basic Research to Enable Agricultural Development (BREAD) Program under Grant no. MCB-1543922.

References

Abdou, Y.A.M., W.C. Gregory, and W.E. Cooper. 1974. Sources and nature of resistance to *Cercospora arachidicola* Hori and *Cercosporidium personatum* (Berk. and Curt.) Deighton in *Arachis* species. Peanut Sci. 1:6–11. doi:10.3146/i0095-3679-1-1-3

Bertioli, D.J., S.B. Cannon, L. Froenicke, G. Huang, A.D. Farmer, E.K.S. Cannon, et al. 2016. The genome sequences of *Arachis duranensis* and *Arachis ipaënsis*, the diploid ancestors of cultivated peanut. Nat. Genet. 48:438–446. doi:10.1038/ng.3517

Bertioli, D.J., M. Moretzsohn, L.H. Madsen, N. Sandal, S. Leal-Bertioli, P.M. Guimaraes, et al. 2009. An analysis of synteny of *Arachis* with *Lotus* and *Medicago* sheds new light on the structure, stability and evolution of legume genomes. BMC Genomics 10:45. doi:10.1186/1471-2164-10-45

Canady, M.A., Y. Ji, and R.T. Chetelat. 2006. Homeologous recombination in *Solanum lycopersicoides* introgression lines of cultivated tomato. Genetics 174:1775–1788. doi:10.1534/genetics.106.065144

Carlson, C.S., M.A. Eberle, M.J. Rieder, Q. Yi, L. Kruglyak, and D.A. Nickerson. 2004. Selecting a maximally informative set of single-nucleotide polymorphisms for association analyses using linkage disequilibrium. Am. J. Hum. Genet. 74:106–120. doi:10.1086/381000

Chetelat, R.T., V. Meglic, and P. Cisneros. 2000. A genetic map of tomato based on BC_1 *Lycopersicon esculentum* × *Solanum lycopersicoides* reveals overall synteny but suppressed recombination between these homeologous genomes. Genetics 154:857–867.

Clevenger, J.P., W. Korani, Y. Chu, and P. Ozias-Akins. 2017. Axiom_Arachis2 genotyping resource for peanut. Proc. Am. Peanut Res. Educ. Soc. 49:77.

Company, M., H.T. Stalker, and J.C. Wynne. 1982. Cytology and leafspot resistance in *Arachis hypogaea* × wild species hybrids. Euphytica 31:885–893. doi:10.1007/BF00039228

Finch, R.A. 1983. Tissue-specific elimination of alternative whole parental genomes in one barley hybrid. Chromosoma 88:386–393. doi:10.1007/BF00285861

Foncéka, D., T. Hodo-Abalo, R. Rivallan, I. Faye, M.N. Sall, O. Ndoye, et al. 2009. Genetic mapping of wild introgressions into cultivated peanut: A way toward enlarging the genetic basis of a recent allotetraploid. BMC Plant Biol. 9:103. doi:10.1186/1471-2229-9-103

Garcia, G.M., H.T. Stalker, and G. Kochert. 1995. Introgression analysis of an interspecific hybrid population in peanuts (*Arachis hypogaea* L.) using RFLP and RAPD markers. Genome 38:166–176. doi:10.1139/g95-021

Garcia, G.M., H.T. Stalker, E. Shroeder, and G. Kochert. 1996. Identification of RAPD, SCAR, and RFLP markers tightly linked to nematode resistance genes introgressed from *Arachis cardenasii* into *Arachis hypogaea*. Genome 39:836–845. doi:10.1139/g96-106

Gernand, D., T. Rutten, A. Varshney, M. Rubtsova, S. Prodanovic, C. Brüß, et al. 2005. Uniparental chromosome elimination at mitosis and interphase in wheat and pearl millet crosses involves micronucleus formation, progressive heterochromatinization, and DNA fragmentation. Plant Cell 17:2431–2438. doi:10.1105/tpc.105.034249

Guo, Y., S. Khanal, S. Tang, J.E. Bowers, A.F. Heesacker, N. Khalilian, et al. 2012. Comparative mapping in intraspecific populations uncovers a high degree of macrosynteny between A- and B- genome diploid species of peanut. BMC Genomics 13:608. doi:10.1186/1471-2164-13-608

Halward, T.M., H.T. Stalker, E.A. Larue, and G.A. Kochert. 1991. Genetic variation detectable with molecular markers among unadapted germplasm resources of cultivated peanut and related wild species. Genome 34:1013–1020. doi:10.1139/g91-156

Halward, T.M., H.T. Stalker, E.A. Larue, and G.A. Kochert. 1992. Use of single-primer DNA amplifications in genetic studies of peanut (*Arachis hypogaea* L.). Plant Mol. Biol. 18:315–325. doi:10.1007/BF00034958

Hancock, W.G., V. Kuraparthy, S.P. Kernodle, and R.S. Lewis. 2015. Identification of maternal haploids of *Nicotiana tabacum* aided by transgenic expression of green fluorescent protein: Evidence for chromosome elimination in the *N. tabacum* × *N. africana* interspecific cross. Mol. Breed. 35:179. doi:10.1007/s11032-015-0372-8

Husted, L. 1936. Cytological studies of the peanut, *Arachis*. II. Chromosome number, morphology and behavior, and their application to the problem of the origin of the cultivated forms. Cytologia (Tokyo) 7:396–423. doi:10.1508/cytologia.7.396

Isleib, T.G., P.W. Rice, R.W. Mozingo, and H.E. Pattee. 1999. Registration of 'Gregory' peanut. Crop Sci. 39:1526. doi:10.2135/cropsci1999.0001rcv

Isleib, T.G., P.W. Rice, R.W. Mozingo, II, S.C. Copeland, J.B. Graeber, B.B. Shew, et al. 2006. Registration of N96076L peanut germplasm. Crop Sci. 46:2329–2330. doi:10.2135/cropsci2005.12.0479

Kasha, K.J., and K.N. Kao. 1970. High frequency haploid production in barley (*Hordeum vulgare* L.). Nature 225:874–876. doi:10.1038/225874a0

Kochert, G., H.T. Stalker, M. Gimenes, L. Galgaro, C. Romero-Lopes, and K. Moore. 1996. RFLP and cytogenetic evidence on the origin and evolution of allotetraploid domesticated peanut, *Arachis hypogaea* (Leguminosae). Am. J. Bot. 83:1282–1291. doi:10.1002/j.1537-2197.1996.tb13912.x

Krapovickas, A., and W.C. Gregory. 2007. Taxonomy of the genus *Arachis* (Leguminosae) (English translation of 1994 Bonplandia 16:1–205 by D.E. Williams and C.E. Simpson). Bonplandia 16:1–205.

Leal-Bertioli, S., K. Shirasawa, B. Abernathy, M. Moretzsohn, C. Chavarro, J. Clevenger, et al. 2015. Tetrasomic recombination is surprisingly frequent in allotetraploid *Arachis*. Genetics 199:1093–1105. doi:10.1534/genetics.115.174607

Lyerly, J.H., H.T. Stalker, J.W. Moyer, and K. Hoffman. 2002. Evaluation of *Arachis* species for resistance to tomato spotted wilt virus. Peanut Sci. 29:79–84. doi:10.3146/pnut.29.2.0001

McDonald, B.A., and C. Linde. 2002. Pathogen population genetics evolutionary potential, and durable resistance. Annu. Rev. Phytopathol. 40:349–379. doi:10.1146/annurev.phyto.40.120501.101443

Moretzsohn, M.C., E.G. Gouvea, P.W. Inglis, S.C.M. Leal-Bertioli, J.F.M. Valls, and D.J. Bertioli. 2013. A study of the relationships of cultivated peanut (*Arachis hypogaea*) and its most closely related wild species using intron sequences and microsatellite markers. Ann. Bot. 111:113–126. doi:10.1093/aob/mcs237

Nagy, E.D., Y. Chu, Y. Guo, S. Khanal, S. Tang, Y. Li, et al. 2010. Recombination is suppressed in an alien introgression in peanut harboring *Rma*, a dominant rootknot nematode resistance gene. Mol. Breed. 26:357–370. doi:10.1007/s11032-010-9430-4

North Carolina Cooperative Extension. 2017. 2017 Peanut information. North Carolina State Univ., Raleigh.

R Core Team. 2017. R: A language and environment for statistical computing. R Found. Stat. Comput., Vienna.

Ramos, M.L., G. Fleming, Y. Chu, Y. Akiyama, M. Gallo, and P. Ozias-Akins. 2006. Chromosomal and phylogenetic context for conglutin genes in *Arachis* based on genomic sequence. Mol. Genet. Genomics 275:578–592. doi:10.1007/s00438-006-0114-z

Ravi, M., and S.W.L. Chan. 2010. Haploid plants produced by centromere-mediated genome elimination. Nature 464:615–618. doi:10.1038/nature08842

Ravi, M., and S.W.L. Chan. 2013. Centromere-mediated generation of haploid plants. In: J. Jiang and J.A. Birchler, editors, Plant centromere biology. John Wiley & Sons, New York. p. 169–181. doi:10.1002/9781118525715.ch13

RStudio Team. 2017. RStudio: Integrated development for R. RStudio, Boston, MA.

Santana, S.H., and J.F.M. Valls. 2015. *Arachis veigae* (Fabaceae), the most dispersed wild species of the genus, and yet taxonomically overlooked. Bonplandia 24:139–150.

SAS Institute. 2013a. JMP Genomics for Windows. Release 8.0. SAS Inst., Cary, NC.

SAS Institute. 2013b. The SAS system for Windows. Release 9.4. SAS Inst., Cary, NC.

Seijo, J.G., G.I. Lavia, A. Fernandez, A. Krapovickas, D.A. Ducasse, D.J. Bertioli, and E.A. Moscone. 2007. Genomic relationships between the cultivated peanut (*Arachis hypogaea-Leguminosae*) and its close relatives revealed by double GISH. Am. J. Bot. 94:1963–1971. doi:10.3732/ajb.94.12.1963

Seijo, J.G., G.I. Lavia, A. Fernandez, A. Krapovickas, D. Ducasse, and E.A. Moscone. 2004. Physical mapping of 5S and 18S–25S rRNA genes by FISH as evidence that *Arachis duranensis* and *A. ipaënsis* are the wild diploid progenitors of *A. hypogaea* (*Leguminosae*). Am. J. Bot. 91:1294–1303. doi:10.3732/ajb.91.9.1294

Simpson, C.E. 1991. Pathways for introgression of pest resistance into *Arachis hypogaea* L. Peanut Sci. 18:22–26. doi:10.3146/i0095-3679-18-1-8

Simpson, C.E. 2001. Use of wild *Arachis* species/introgression of genes into *A. hypogaea* L. Peanut Sci. 28:114–116. doi:10.3146/i0095-3679-28-2-12

Singh, A.K., and J.P. Moss. 1984. Utilization of wild relatives in the genetic improvement of *Arachis hypogaea* L. Part 5. Genome analysis in section *Arachis* and its implications in gene transfer. Theor. Appl. Genet. 68:355–364. doi:10.1007/BF00267889

Smartt, J., and W.C. Gregory. 1967. Interspecific cross-compatibility between the cultivated peanut *Arachis hypogaea* L. and other species of the genus *Arachis*. Oleagineux 22:455–459.

Stalker, H.T. 1984. Utilizing *Arachis cardenasii* as a source of *Cercospora* leafspot resistance for peanut improvement. Euphytica 33:529–538. doi:10.1007/BF00021154

Stalker, H.T. 1985. Cytotaxonomy of *Arachis*. In: J.P. Moss, editor, Proceedings of the International Workshop on Cytogenetics of *Arachis*, Patancheru, India. 31 Oct.–2 Nov. 1983. ICRISAT, Patancheru. p. 65–79.

Stalker, H.T. 1997. Peanut (*Arachis hypogaea* L.). Field Crops Res. 53:205–217. doi:10.1016/S0378-4290(97)00032-4

Stalker, H.T. 2017. Utilizing wild species for peanut improvement. Crop Sci. 57:1102–1120. doi:10.2135/cropsci2016.09.0824

Stalker, H.T., and M.K. Beute. 1993. Registration of four interspecific peanut germplasm lines resistant to *Cercospora arachidicola*. Crop Sci. 33:1117. doi:10.2135/cropsci1993.0011183X003300050064x

Stalker, H.T., M.K. Beute, B.B. Shew, and K.R. Barker. 2002a. Registration of two root-knot nematode resistant peanut germplasm lines. Crop Sci. 42:312–313. doi:10.2135/cropsci2002.3140

Stalker, H.T., M.K. Beute, B.B. Shew, and T.G. Isleib. 2002b. Registration of five leaf spot-resistant peanut germplasm lines. Crop Sci. 42:314–316. doi:10.2135/cropsci2002.3140

Stalker, H.T., and R.E. Lynch. 2002. Registration of four insect-resistant peanut germplasm lines. Crop Sci. 42:313–314. doi:10.2135/cropsci2002.3130

Stalker, H.T., and J.P. Moss. 1987. Speciation, cytogenetics, and utilization of *Arachis* species. Adv. Agron. 41:1–40. doi:10.1016/S0065-2113(08)60801-9

Stalker, H.T., and J.C. Wynne. 1979. Cytology of interspecific hybrids in section *Arachis* of peanuts. Peanut Sci. 6:110–114. doi:10.3146/i0095-3679-6-2-12

Stalker, H.T., J.C. Wynne, and M. Company. 1979. Variation in progenies of an *Arachis hypogaea* × diploid wild species hybrid. Euphytica 28:675–684. doi:10.1007/BF00038934

Subrahmanyam, N.C., and K.J. Kasha. 1973. Selective chromosomal elimination during haploid formation in barley following interspecific hybridization. Chromosoma 42:111–125. doi:10.1007/BF00320934

Subrahmanyam, P., A.M. Nolt, B.L. Reddy, D.V.R. Reddy, and D. McDonald. 1985. Resistance to groundnut diseases in wild *Arachis* species. In: J.P. Moss, editor, Proceedings of the International Workshop on Cytogenetics of *Arachis*, Patancheru, India. 31 Oct.–2 Nov. 1983. ICRISAT, Patancheru. p. 49–55.

Tallury, S.P., T.G. Isleib, S.C. Copeland, P. Rosas-Anderson, M. Balota, D. Singh, and H.T. Stalker. 2013. Registration of two multiple disease-resistant peanut germplasm lines derived from *Arachis cardenasii* Krapov. & W.C. Gregory, GKP 10017. J. Plant Reg. 8:86–89. doi:10.3198/jpr2013.04.0017crg

Tallury, S.P., R. Srinivasan, P. Chu, W. Park, and T. Ranney. 2014. Characterization of Gregory × *Arachis diogoi* (GK 10602; PI 276235) interspecific hybrid population. In: Proceedings of the 7th International Conference on Advances in *Arachis* through Genomics and Biotechnology (AAGB), Savannah, GA. 11–14 Nov. 2014. PeanutBase. p. 7–8. https://www.peanutbase.org/files/AAGB/Abstracts_--_aagb-2014_--_final_11-4-2014.pdf (accessed 18 Nov. 2019).

Thermo Fisher Scientific. 2016. Axiom Analysis Suite version 3.0.1.4 user guide. Publ. 703307, Version 7. Affymetrix, Santa Clara, CA. https://downloads.thermofisher.com/Axiom_Analysis_Suite_v_4.0.1_User_Guide.pdf (accessed 18 Jan. 2019).

Valls, J.F.M., L.C. Costa, and A.R. Custodio. 2013. A novel trifoliolate species of *Arachis* (Fabaceae) and further comments on the taxonomic section *Trierectoides*. Bonplandia 22:91–97.

Valls, J.F.M., and C.E. Simpson. 2005. New species of *Arachis* (Leguminosae) from Brazil, Paraguay and Bolivia. Bonplandia 14:35–63.

Valls, J.F.M., and C.E. Simpson. 2017. A new species of *Arachis* (Fabaceae) from Mato Grosso, Brazil, related to *Arachis matiensis*. Bonplandia 26:143–149. doi:10.30972/bon.2622575

van Berloo, R. 2008. GGT 2.0: Versatile software visualization and analysis of genetic data. J. Hered. 99:232–236. doi:10.1093/jhered/esm109