From: Bill Rooney
To: "Les Kuhlman"
Subject: FW: 09-105 - Revise Manuscript
Date: Thursday, August 27, 2009 7:17:00 AM
Attachments: Genome 09-105 Revision.doc

Les:

I realized I can send this to your Pioneer address as well. So in case you haven't yet, here it is.

I've made corrections and resubmitted the revised version (I've attached that to this e-mail).

I also have all of the permission to copyright forms (except yours) signed and I'll send those in.

What I don't know - they have a section for adding good files for images and tables. Do you have those files or should they simply use the revised manuscript? (ie, in the last manuscript, what did you send them?) If they are different files, do you have those files and can you upload them?

Regards,

Bill

P.S. I have approval to release Tx3361, so I am reworking the manuscript and submitting it for release. Before I submit, I'll send the registration manuscript up to you for approval.

Dr. William L. Rooney
Professor, Sorghum Breeding and Genetics
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979 845 2151

-----Original Message-----
From: Editorial Office [mailto:genome@yorku.ca]
Sent: Thursday, August 06, 2009 8:32 PM
To: wlr@tamu.edu
Subject: 09-105 - Revise Manuscript

Dear William Rooney ,

Re: 09-105
Early-generation Germplasm Introgression from Sorghum macrospermum into Sorghum (S. bicolor) Les LCK Kuhlman, Byron BLB Burson, David Stelly, Patricia Klein, Robert R Klein, Harold James H.J. Price, and William WLR Rooney

Perry Gustafson has received and assessed reviewer comments for your manuscript. Based on the reviewer comments, Perry Gustafson recommends you submit a revised manuscript.

To submit a revised manuscript, log on to OSPrey at https://endeavour.cisti.nrc.ca/publisher/access.view?journalCode=GENOME and click on "Author" in the "Your Work Areas" box. Please DO NOT submit a new manuscript as this will lead to delays.

Below I have printed the reviewer comments and the comments of Perry Gustafson.

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Sincerely,
Alistair Coulthard
Assistant to the Editor
GENOME
e-mail: genome@yorku.ca

Associate Editor's Comments:

I agree with the reviewer in that this is a very well written manuscript. However, it does need tot be carefully edited by the authors to make several small corrections as noted in the review.

Review 1
Questions/Answers

Q.There are four general questions for recommendation:
A.Accept as it stands

Comments

These are my general/specific comments:

The manuscript is well written. Properly methodology and protocol were followed in conducting the research. Conclusions drawn are proper.

The research adds new knowledge on the potential to introgress genes from other Sorghum species into S. bicolor.

Manuscript is acceptable for publication as submitted.

The reference Sharma (1999) on page 6 is not listed in the References.

Huelgas et al., reference - location is Tamworth, not Tomworth. (See Franzmann and Hardy)

Introgression Breeding using *S. macrospermum* and Analysis of Recovered Germplasm

Les C. Kuhlman, Byron L. Burson, David M. Stelly, Patricia E. Klein, Robert R. Klein, H.J. Price, and William L. Rooney

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ABSTRACT

Sorghum has been improved by public and private breeding programs utilizing germplasm mostly from within the species *Sorghum bicolor*. Recently, hybridization with an Australian species, *S. macrospermum* (AAB_1B_1YYZZ), has been demonstrated and the genomic relationship to *S. bicolor* (AAB_1B_1) shown to be partially compatible. For this species to be potentially useful to sorghum improvement programs, there must be documented introgression into an *S. bicolor* background. Fifteen BC_1F_1 progeny were recovered using the interspecific hybrid as a female and embryo rescue. In these progeny, chromosome numbers ranged from 35 – 70 and all were essentially male sterile. Repeated backcrossing with *S. bicolor* pollen, produced [redacted] seed on 3 of the 15 [redacted] plants. [redacted] progeny had varying levels of male fertility; selfed seed set ranged from 0 – 95% with only 2 being completely male sterile. Using AFLP and SSR markers, genomic introgression of *S. macrospermum* ranged from 0 – 18.6%. Cytogenetic analysis of 19 individuals revealed chromosome numbers were 20, except for a single backcross which had 21 chromosomes. Molecular cytogenetic analysis confirmed the presence of recombinant introgression chromosomes as well as alien addition and alien substitution chromosomes within the BC_2F_1s.

INTRODUCTION

Sorghum (*S. bicolor* [L.] Moench) is an important food and feed crop around the world. The 2006 U.S. grain sorghum crop was valued at approximately $715 million (USDA, 2006) and worldwide is the 5th most grown cereal grain. Plant breeders continuously improve the crop for yield potential, drought tolerance, disease and insect resistance, and other biotic and abiotic stresses. Genetic variation is the lifeblood of plant breeding so identification of useful new sources is a worthwhile endeavor.

Taxonomically, the genus *Sorghum* is separated in to 5 sections: *Eusorghum*, *Chaetosorghum*, *Heterosorghum*, *Parasorghum*, and *Stiposorghum* (Garber, 1950; de Wet, 1978). The cultivated species is grouped within section *Eusorghum* along with *S. propinquum* and the noxious weed *S. halepense*. Genetic improvements in sorghum have been made by utilizing genetic variation from within the primary gene pool, which contains all of the germplasm in the three subspecies of *S. bicolor*: ssp. *arundicum*, *bicolor*, and *drumondii* (de Wet, 1978; Cox et al., 1984; Duncan et al., 1991). The secondary gene pool is composed of the remaining two species in *Eusorghum*. Crosses between sorghum and *S. propinquum* are easily made, meiosis is normal in the interspecific hybrids, and progeny are fertile, but there has been little to no use of this germplasm in applied sorghum improvement (Wooten, 2001). Hybrids between sorghum and *S. halepense* are more difficult to produce but still possible. Most efforts in utilizing *S. halepense* as a genetic resource have been devoted to developing perennial grain crops (Piper and Kulakow, 1994; Cox et al., 2002; Dweikat, 2005). The tertiary gene pool contains the 17 remaining species within the four other sections. Until

recently, this gene pool was completely inaccessible as no hybrids had ever been recovered despite numerous efforts (Karper and Chisholm, 1936; Ayyanger and Ponnaiya, 1941; Garber, 1950; Endrizzi, 1957; Tang and Liang, 1988; Wu, 1990; Sun et al., 1991; Huelgas et al., 1996).

The cause of reproductive isolation between sorghum and the tertiary gene pool was unknown until Hodnett et al., (2005) determined that it was due to pollen-pistil incompatibilities. Pollen tube growth of wild species was inhibited in the stigma and style which prevented successful fertilization. The reproductive barriers proved to be strong but not complete as Price et al., (2005) finally recovered one interspecific hybrid between cytoplasmic male-sterile (CMS) sorghum and *S. macrospermum*. The efficiency of producing this hybrid improved dramatically by using a *S. bicolor* genotype homozygous for the *iap* allele. The *Iap* locus (*I*nhibition of *A*lien *P*ollen) controls a pistil barrier that prevents foreign species pollen tube growth; whereas, the recessive genotype (*iap iap*) allows pollen tube growth of maize as well as wild sorghum species (Laurie and Bennett, 1989; Price et al., 2006). Price et al., (2006) recovered hybrids between sorghum and *S. macrospermum*, *S. nitidum*, and *S. angustum* but only hybrids with *S. macrospermum* survived to maturity.

S. macrospermum (2*n* = 40) is the only member of the *Chaetosorghum* section and it is native to the Katherine area in the Northern Territory of Australia (Lazarides et al., 1991). While this species does not possess any obvious agronomically desirable traits, it does have significant pest resistance. It is either a non-host or has ovipositional non-preference to sorghum midge (*Stenodiplosis sorghicola* Coquillett) (Franzmann and

Hardy, 1996; Sharma and Franzmann, 2001). It is not susceptible to sorghum downy mildew (*Peronosclerospora sorghi* Weston and Uppal (Shaw)) (Kamala et al., 2002) and has high tolerance to shoot fly (*Atherigona soccata* Rond.) (Sharma et al., 2005). These beneficial traits, as well as the possibility that it holds other valuable unique genetic variation, make it attractive to use in an introgression breeding program.

Until recently, the genomic relationship between *S. macrospermum* and *S. bicolor* was not known. Several authors have described *S. bicolor* ( ) has an ancient tetraploid; its genomic formula was derived by analyzing meiosis in hybrids with *S. halepense* ( ) (Hadley, 1953; Celerier, 1958; Tang and Liang, 1988). Meiotic chromosome pairing behavior in interspecific hybrids between *S. bicolor* and *S. macrospermum* revealed that moderate levels of allosyndetic recombination occurred and the genomic formula AAB_1B_1YYZZ was proposed for *S. macrospermum* ($2n = 8x = 40$) (Kuhlman et al., 2008). Allosyndetic recombination was observed in subgenomes A and B_1, but the frequency was 2.5 times higher in subgenome A. The authors attempted to produce backcrosses using the interspecific hybrid as a male, but were not successful.

The tertiary gene pool species *S. macrospermum* is now available to plant breeders because hybrids can now be recovered by using specific *S. bicolor* germplasm (*iap iap*). The sorghum and wild species genomes undergo moderate levels of allosyndetic recombination; therefore, recovering introgression in backcross progeny is likely (Kuhlman et al. 2008). The remaining obstacle to using this species in an introgression program is determining how to recover backcrosses. The objectives of this

research were to produce 2*n* = 20 introgression germplasm through backcrossing and to analyze introgression content in backcross progeny molecularly and cytologically.

MATERIALS AND METHODS

Plant Material

Interspecific hybrids were produced by hand emasculating 'NR481', the *S. bicolor* parent, and pollinating it with the wild species [redacted]). Female plants set approximately 25% hybrid seed, which had shrunken endosperm. Approximately 60% of hybrid seeds germinated on agar germination media and were transplanted into soil in small pots in a greenhouse during April, 2005 in College Station, TX. They were transplanted as growth demanded up to a final pot size of 15 gallons. Interspecific hybrids were tall (> 4.5m) and photoperiod sensitive (initiating anthesis in September). Backcrosses were made using pollen from both the recurrent parent [redacted]

Embryo rescue was necessary to recover backcrosses and was performed 15 to 20 days after pollination. Enlarged ovaries were removed from the florets and surface sterilized in 30% bleach for 20 minutes. The soft pericarp tissue was removed and the immature embryos were placed in sealed Petri dishes on culture medium containing Murashige-Skoog basal salts and vitamins (Murashige and Skoog, 1962) supplemented with 10mg L^{-1} glycine, 10mg L^{-1} L-arginine, 10mg L^{-1} L-tyrosine, 100mg L^{-1} inositol, and [redacted] solidified with [redacted] plant tissue culture grade agar (Sharma, 1999). Dishes were placed in a growth chamber with 16 h light/8 h dark at 24ºC.

Germinated embryos with good root growth and 2-3 leaves were removed from the media and transplanted into a fine texture soil mixture in pots. These were placed in a plastic tray with a clear dome lid inside the growth chamber with wet paper towels to ensure high humidity. As plants grew they were hardened off and transferred to the greenhouse.

Germplasm Evaluation

Male gamete viability was estimated by collecting anthers from flowering plants and macerating them in a drop of 1% I_2-KI stain on a glass slide. Slides were analyzed under a microscope, pollen grains were counted and classified as fully stained, greater than 50% stained, less than 50% stained, and unstained. Plant height was measured in inches from the soil surface to the tip of the mature panicle. Some plants were also characterized for plant color, seed color, presence of awns, mid-rib type, days to 50% anthesis, and seed set. Field evaluation of selected BC_2F_1 progeny from family 101 was carried out in Weslaco, TX in fall, 2006. Plants were self pollinated and at harvest evaluated for plant height and seed color. Specific measure of seed set was not taken although no plants were identified as sterile. Evaluation of BC_2F_1 progeny from all three families was carried out in a greenhouse in winter 2006 in College Station, TX.

Molecular Marker Evaluation

DNA was extracted from backcross progeny and their parents using the FastDNA Spin Kits (MP Biomedicals, Solon, OH). AFLP templates, using both *EcoRI*/*MseI* and

PstI/*MseI* restriction enzyme combinations, were created using a modified procedure from Vos et al., (1995). The AFLP template, preamplification, and selective amplification reactions of the *EcoRI*/*MseI* and *PstI*/*MseI* fragments were as described by Klein et al (2000) and Menz et al (2002), respectively. Twenty *Pst*/*Mse* and 12 *EcoRI*/*Mse* AFLP primer combinations were used to amplify fragments in the DNA samples. IRD-labeled SSR primers, obtained from LI-COR (LI-COR Inc., Lincoln, NE), were used in amplification reactions as previously described (Klein et al., 1998). Twenty-eight SSR primer combinations were run on the DNA samples, but only 11 (39%) showed transferability by producing a band in the wild species. Amplification products were analyzed on a LI-COR model 4200 dual-dye automated DNA sequencing system. Electrophoresis conditions were as described by Klein et al. (2000). Gels were scored manually, AFLP bands that were present in *S. macrospermum* and absent in the recurrent *S. bicolor* parents were scored as unique. Unique bands that were also shared by backcross progeny were scored as introgression bands. The percent introgression was calculated by dividing the number of introgression bands a particular backcross produced by the total number of unique *S. macrospermum* bands. This number is an estimate of the amount of the *S. macrospermum* genome that is present in the backcross progeny. Since backcrosses were produced using the female interspecific hybrid gamete there is no question as their authenticity as true backcrosses, thus introgression bands can be interpreted as actually representing transfer of *S. macrospermum* DNA into the progeny.

Cytogenetic Evaluation

Somatic chromosome spreads were prepared from root tips using a modified procedure from Andras et al. (1999). Root tips were harvested into a saturated aqueous solution of α-bromonapthalene for 1.75 h at room temperature in the dark. Pretreated root tips were fixed in 95% ethanol/glacial acetic acid (4:1 v/v) for 24 h and stored in 70% ethanol. Root tips were graded based on size standards of 0.0 – 1.0 mm. The terminal 1mm of several same sized root tips were dissected into a 0.5ml epitube, rinsed in water several times, hydrolyzed for 10 min in 0.2*M* HCl, and rinsed 10 min in distilled water. Cell walls were digested by adding 100ul of an aqueous solution of 3% cellulase (Onozika R-10, Yakult Honsha Co. Ltd., Tokyo) and 1% pectolyase Y-23 (Seishin Corp., Tokyo) at pH 4.5 for 1-2 h at 37ºC. Digestion times were based on empirically determined values for a particular size standard. Digestion was stopped by adding 400ul distilled water and centrifuging the cell suspension at 2500rpm (~400G) for 10 min. Using a drawn glass pipette, the supernatant was removed being careful not to disturb the pellet of cells. The cells were washed with water and centrifuged at 2500rpm for 10 min., twice. After removal of the final wash water, 400ul of methanol/glacial acetic acid (4:1 v/v) was used to wash the cells followed by centrifugation at 2500rpm for 10 min., twice. After the final wash, all but ~50ul of the fixative was removed. The cells were resuspended in the remaining fixative, 2-8ul drops were placed on clean glass slides suspended over wet filter paper and allowed to dry. For chromosome counts, slides were stained with Azure Blue, made permanent with Permount, and analyzed with a Zeiss Universal II microscope (Carl Zeiss Inc., Gottingen, Germany). A minimum of four

193 quality spreads of highly condensed chromosomes was used to determine the somatic
194 chromosome number of individual plants.
195 Fluorescent and Genomic *in situ* hybridization (FISH and GISH) were used to
196 visualize introgression in backcross progeny. Plasmid CEN38 was used as a FISH probe
197 to visually differentiate *S. bicolor* subgenomes A and B_1 (Gomez et al., 1998; Zwick et
198 al., 2000). Genomic DNA of *S. macrospermum* and *S. bicolor* were used as GISH
199 probes to detect introgression DNA in the backcrosses and to determine whether the
200 chromosomes were recombinant. Detection of probes followed a modified protocol of
201 Jewell and Islam-Faridi (1994), as described by Hanson et al. (1995) and Kim et al.
202 (2002). Purified probe DNA was nick-translated with digoxigenin-11-dUTP or biotin-
203 16-dUTP (Roche Diagnostics, Indianapolis, IN). Slides with somatic chromosome
204 spreads were prepared as described above. Chromosomes on glass slides were denatured
205 in 70% formamide in 2X SSC for 1.5 min at 70ºC, then dehydrated in 70 (-20ºC), 85
206 (RT), 95 (RT), and 100% (RT) ethanol, for 2 min each. The hybridization mixture (25ul
207 per slide) contained 50ng labeled probe DNA, 50% formamide and 10% dextran sulfate
208 in 2X SSC. The hybridization mixture was denatured for 10 min at 95ºC and chilled on
209 ice. It was then added to the slide, sealed with rubber cement around a glass coverslip
210 and incubated overnight at 37ºC. Following incubation, the slides were washed at 40ºC
211 in 2X SSC and room temperature in 4X SSC plus 0.2% Tween-20, for 5 min each.
212 Slides were blocked with 5% (w/v) BSA in 4X SSC plus 0.2% Tween-20 at room
213 temperature. The digoxigenin and biotin-labeled probes were detected with CY3™-
214 conjugated anti-digoxigenin anti-body and fluorescein isothiocyanate (FITC)-conjugated

streptavidin, respectively. Slides were washed in 37ºC 4X SSC plus 0.2% Tween-20. Chromosomes were counterstained with 25ul DAPI with Vectashield® (Vector Laboratories, Burlingame, CA). Slides were viewed through an Olympus AX-70 epifluorescence microscope and images captured with a Macprobe® v4.2.3 imaging system (Applied Imaging Corp., Santa Clara, CA).

RESULTS AND DISCUSSION

Breeding Methodology, Cytology, and Germplasm Phenotypic Evaluation

Interspecific Hybrids: Twenty interspecific hybrids were grown and their identity was confirmed by morphology and chromosome number ($2n = 30$). At maturity, hybrids flowered but anthers were non-dehiscent. Normal I_2-KI staining pollen grains were rare and F_2 seed did not develop on 15 selfed panicles (approximately 3,000 florets). Previous attempts to recover backcross progeny using the male hybrid gamete were difficult and inconclusive (Kuhlman et al. 2008). Interspecific hybrid panicles were pollinated with *S. bicolor* pollen, mostly from [redacted] but a few also with [redacted]. Backcross seed development was rare: a single seed with well developed endosperm was observed but it was not viable. Thus, embryo rescue was used to recover backcross progeny. In total, 7009 florets were pollinated and dissected revealing 86 (1.2%) with embryo development of which 15 (0.2%) survived into adult [redacted] plants (Figure 1).

[redacted] *plants:* All [redacted] had awns and red plant color but varied in their height and vigor (Table 1). Most [redacted] plants had little to no male fertility with non-dehiscent anthers and non-viable pollen; the seed that was produced was all red in pericarp color

(Table 1). Most [redacted] plants were backcrossed using [redacted] pollen; occasionally [redacted] was used when adequate supplies of [redacted] pollen were unavailable. Embryo rescue was not needed as 3 [redacted] plants ([redacted]) set viable backcross seed (Table 1). Two other plants, [redacted] produced a single backcross seed that was not viable (Table 1).

[redacted] was morphologically distinct from the others; it had wider leaves, larger florets, and had features reminiscent of [redacted] marker data confirmed that [redacted] was derived from [redacted] fertilization of the interspecific hybrid. Phenotypic and molecular data confirmed that [redacted] and [redacted] resulted from fertilization by [redacted]. Both of these [redacted] produced significantly less backcross seed than did [redacted] (Table 1). The increased seed set in [redacted] could be due to increased heterozygosity resulting from its mixed pedigree.

Chromosome numbers in the [redacted] plants ranged from [redacted] (Table 1, Figure 1). Such high chromosome numbers resulted from irregular meiosis in the interspecific hybrid (Kuhlman et al. 2008). [redacted] plants with chromosome numbers between 35 and 39 likely resulted from transmission of [redacted] chromosomes through the female gamete and 10 chromosomes through the *S. bicolor* gamete. Transmission of [redacted] chromosomes from plants with [redacted] is best explained by the formation of a restitution nucleus composed of the univalents during meiosis. Under this hypothesis, chromosomes would pair at meiosis, and those undergoing recombination would form bivalents at metaphase I and subsequently separate and move to the spindle poles. The remaining chromosomes would form univalents, some of which might distribute

themselves to the poles via spindle attachment, while the others would remain at the metaphase I plate and other intermediate positions. In cells with a pole-to-pole distribution of univalents, a restitution nucleus would sometimes form between the two poles, and the product would contain all or most chromosomes. Meiosis II typically conserves chromosome numbers of meiosis I products, so variable chromosome numbers among restitution and partial-restitution products from meiosis I would translate to megagametophytes with various chromosome numbers. Restitution nuclei have been implicated in transmission of univalents in multiple species (Singh, 2003). The two plants with [redacted] and [redacted] chromosomes may have been produced due to meiotic irregularities (Singh, 2003) resulting in tetraploid ([redacted]) female gametes. Parthenogenesis of such a "4n" egg would result in [redacted] progeny or fertilization of such an egg would result in [redacted] progeny. [redacted]), is hypothesized to be a naturally produced allododecaploid. It displayed slow growth and very stiff leaves, and complete sterility; backcrosses were not recovered.

[redacted] *amilies*: Three [redacted] families consisting of [redacted] seed from the three partially fertile [redacted] were planted and evaluated. Pollen samples were taken from plants of each family and scored for pollen stainability. All three BC_2 families had significantly lower mean pollen stainability than [redacted] Family [redacted] had higher pollen stainability than [redacted] which were not different (Table 2). [redacted] families [redacted] displayed significantly lower seed set ([redacted] than family [redacted] which were not different (Table 2). The vastly lower

seed set from families made obtaining selfed seed difficult and limited the evaluation of the generation.

Chromosome number for plants within family plants analyzed; one plant was Two plants each from families had chromosomes (Table 2). progeny (were produced without embryo rescue from parents that contained chromosomes. Whereas the restitution nucleus conferred survivability to the rescued embryos, it appears that it was selected against when embryos were not rescued and seeds were produced. Of those surveyed, plants had chromosomes.

All individuals were tall, had red plant and seed color, and a dry midrib like the recurrent *S. bicolor* parent except the in family in which three individuals had white seed color, two individuals had juicy midribs, and one was short (Table 2). These traits are recessively inherited and should not be present in a population of ndividuals whose pollen parent is tall, red seeded, has a dry midrib, and has not been observed to segregate for these traits. Pollen contamination from a different genotype was impossible since no other genotypes were grown in the greenhouse during that time. The simplest explanation is self-pollination, however, fertile pollen was never observed. Parthenogenesis of an unfertilized egg cell is not possible as segregation was observed in selfed progeny (Table 2). Alternatively, gametes could be produced via failed cytokenesis of the dyads during the second stage of meiosis (Singh, 2003). As an example, a pollen mother cell, in this case possessing chromosomes with at metaphase, could produce two dyad

cells with [redacted] chromosomes, assuming the univalents segregated as a restitution nucleus. If cytokenesis failed during meiosis II, the sister chromatids would separate, and following macrogametogenesis form an egg cell with [redacted] chromosomes. If this cell developed into an embryo parthenogenically, it would not necessarily be 100% homozygous since the chromosomes underwent recombination during meiosis I, resulting in the sister chromatids being genetically different. This [redacted] progeny plant could not be differentiated from a selfed plant. Therefore, [redacted] progeny produced from [redacted] are potentially a mix of pedigrees: backcross derived [redacted] selfed [redacted], and parthenogenic progeny from diploid gametes. As separation of all individuals into these classes is not possible, this generation will still be referred to as [redacted]

[redacted] progeny were evaluated for visual expressions of introgression in both the field and greenhouse. Overall, [redacted] progeny deriving from family 101 had adequate seed set and segregated for traits polymorphic between [redacted] and [redacted] such as seed color and plant height. This significant variability in the population made identifying phenotypic evidence of introgression virtually impossible. [redacted] plants in families [redacted] showed one obvious sign of introgression: male-sterility. Female fertility was unaffected as backcross seed set was normal. Partial male sterility in the [redacted] plants in these families was likely caused by *S. macrospermum* introgression and the plants were presumed to be heterozygous for any introgression. [redacted] plants were expected to segregate for male-sterility, but lack of segregation suggests that the [redacted] plants were homozygous for such introgression (Table 2). This could be possible if the

[redacted] were actually the result of selfing, but this is unlikely as stainable pollen was rarely observed. Some form of asexual reproduction, as described for family 101, could also be causing progeny to be homozygous for introgression. There would also have to be high selection pressure for the sterility inducing introgression as all [redacted] plants from these two families produced sterile progeny.

Molecular Marker Analysis of Introgression

The amount of *S. macrospermum* genome that was introgressed into the BC_2 generation was evaluated using AFLP markers. In total, 32 primer combinations produced [redacted] markers unique to *S. macrospermum*. The total amount of *S. macrospermum* genome detected in the [redacted] generation was 26% ([redacted] unique *S. macrospermum* markers). Most introgression bands (82%) were found in single individuals, while 5% were shared by between [redacted]. Each family possessed three types of introgression: unique to that family, shared between two families, and shared by all three families (Figure 2). Estimates for introgression on an individual basis ranged widely from [redacted]% (Table 2), although the amount of introgression did not significantly differ on a family mean basis [redacted]

Eleven of the [redacted] from family [redacted] did not have detectable levels of introgression, while two had the highest levels [redacted] The total amount of introgression detected within family [redacted] was high [redacted], although it was derived primarily from the two outstanding individuals. Introgression was detected in all [redacted] individuals within families [redacted] but the range was narrow, from 0[redacted]

(Table 2). The total amount of introgression detected in families [redacted] and [redacted], respectively. A majority of introgression markers detected in families [redacted] and [redacted] respectively) were present in multiple (4 to 6) individuals within the family, indicating that common introgression sequences were inherited. Thus, inheritance of introgression in these two families does not appear to be random. This data in combination with the phenotypic male-sterility that is expressed by all individuals in these two families suggests there was selection of gametes carrying a common block of introgression. In contrast, almost half of individuals within family [redacted] had no detectable introgression and few markers were present in multiple family members [redacted]. Common introgression was found between the three excluded individuals, but overall introgression in the family 101 appeared random.

The two individuals that were distinctly different from the rest were [redacted], both of which were from family [redacted] and had [redacted] of the *S. macrospermum* genome detected within their DNA. Selected SSR markers were run on these DNA samples to confirm introgression. Two different SSRs confirmed independent introgression of *S. macrospermum* DNA in these plants. [redacted] confirmed introgression in [redacted] but was absent in [redacted], while the opposite confirmation occurred with [redacted] [redacted] are located on [redacted] of the genetic map by Menz et al. (2002) at approximately [redacted] and [redacted], respectively (http://sorgblast3.tamu.edu). SSR markers surrounding these two locations showed that no introgression had occurred in both plants. This indicates that if the introgressed SSR

sequences are on SBI-01, they are part of a small introgression segment. Alternatively, the *S. macrospermum* SSR sequence may not have been homoeologous to SBI-01, and thus be on another *S. bicolor* chromosome, or it was not introgressed into the *S. bicolor* genome at all and be located on a whole *S. macrospermum* addition chromosome.

Molecular Cytogenetic Analysis

Multiple types of *S. macrospermum* introgression were found in the BC_2 generation. [redacted] (18.6% introgression) ($2n = 20$) visibly shows two *S. macrospermum* chromosomes and 18 *S. bicolor* chromosomes in its genome (Figure 3, A). Visualization of the *S. bicolor* genome reveals that the *S. macrospermum* chromosomes are non recombinant (Figure 3, B). The *S. bicolor* chromosomes, evidenced by the CEN38 probe, are 10 from the A subgenome and 8 from the B_1 subgenome. This plant is an example of an alien substitution line: two B_1 *S. bicolor* chromosomes have been replaced with two *S. macrospermum* chromosomes. The introgression detected by molecular markers, including Txp482, is largely located on two *S. macrospermum* alien substitution chromosomes. The cytogenetic evidence, however, cannot disprove the existence of small introgression blocks within the *S. bicolor* genome. This type of introgression has been used extensively in wheat breeding where alien substitution is well tolerated by the genome (Jiang et al., 1994; Jones et al., 1995; Jauhar and Chibbar, 1999). Seed set was slightly lower than the check but still reasonably high (72%). Morphologically this plant appeared to be in the range of that for segregation between [redacted] therefore, no phenotypic trait can

presently be assigned to the alien chromosomes. It is surprising that the plant tolerates this level of alien substitution as *S. bicolor* trisomic lines have been recovered (Schertz, 1966) but monosomic lines have not. This indicates that homoeologous chromosomes from the *S. macrospermum* genome must compensate for the missing *S. bicolor* chromosomes.

GISH using *S. macrospermum* DNA as probe reveals that [redacted] (3.7% introgression) ($2n = 21$) was an alien addition line; it had one non-recombinant *S. macrospermum* chromosome along with 20 *S. bicolor* chromosomes (Figure 3, C and D). The introgression detected using molecular markers in this plant is most likely located on a single *S. macrospermum* chromosome, however, the presence of small introgression blocks cannot be disproven. [redacted] which detected introgression in this plant, most likely is homoeologous to a sequence on the *S. macrospermum* chromosome. This plant displays no deleterious effects of the introgression in that seed set was high (85%) and the plant was vigorous. One potential phenotype influenced by introgression was the presence of normal and shriveled endosperm seeds produced by selfing. The approximate ratio of normal to shriveled seed was not different from a 3:1 ratio ($\chi^2 = 1.12^{ns}$). This would be consistent with reduced seed size for progeny inheriting two copies of the alien chromosome. This presumes, however, that normal segregation of an alien chromosome occurs through both gametes. The fitness of gametes carrying an extra chromosome is normally reduced; thus, the transmission rate of an alien chromosome would also likely be low. It is possible that this phenotype is controlled by

411 the transmission of an alien chromosome, but this hypothesis needs cytological
412 verification.
413 SSR markers [redacted] were detected in [redacted],
414 respectively, but neither marker was present in both plants. This indicates that the alien
415 addition chromosome in 222 is different from both substitution chromosomes in 209.
416 AFLP data is consistent with this hypothesis as only 3 introgression markers are shared
417 out of 98 present in [redacted]209 and 19 present in 222. Both SSR markers map to
418 chromosome 1 in the *S. bicolor* genome, which may indicate that the two detected *S.*
419 *macrospermum* chromosomes are both homoeologous to SBI-01, perhaps the related
420 chromosomes from subgenomes A_m and B_{1m} (Kuhlman et al. 2008). The introgression
421 estimate for 209 is much higher than 222. Introgression estimates were based on AFLP
422 markers which are mostly dominant, therefore being homozygous for an introgression
423 marker does not increase the introgression estimate. Thus, it would be unlikely for
424 [redacted] to contain two homologous *S. macrospermum* substitution chromosomes and
425 still have a five fold increase in estimated introgression. Neither *S. bicolor* nor *S.*
426 *macrospermum* karyotypes show that broad of range for chromosome size, therefore,
427 inheritance of larger homologous chromosomes does not explain the increased
428 introgression (Wu, 1990; Kim et al., 2005a). [redacted] most likely contains two
429 different *S. macrospermum* substitution chromosomes, both of which are different from
430 the addition chromosome in [redacted]
431 GISH using *S. macrospermum* DNA as probe revealed [redacted] (2*n* =
432 20, 20; 1.1% and 0.57% introgression, respectively) both contain two chromosomes with

S. macrospermum introgression. The introgression chromosomes also show hybridization with the *S. bicolor* probe (Fig. 3, F) and strong hybridization with CEN38; therefore, they are members of the A subgenome. Using morphology to identify somatic chromosomes, the introgression sites appear to be located on SBI-01 homologous chromosomes. These two plants are examples of introgression backcrosses, as they contain *S. macrospermum* DNA introgressed into the *S. bicolor* genome. These two plants show phenotypic evidence of introgression like all members of their respective families (102 and 107). Individuals 228 and 244 had low selfed seed set (2.1% and 0.1%, respectively) and all their [redacted] progeny were completely male-sterile. Backcross seed set was normal. This strongly supports the hypothesis that these plants, and possibly all plants in these families, are homozygous for the introgression that they contain.

66% of the AFLP introgression bands in [redacted]244 are common to [redacted] 228. In fact, 17 of 19 [redacted] plants from families 102 and 107 share some common introgression with [redacted]244. A portion of the introgression block present in [redacted] 244 seems to have been preferentially transmitted to most progeny deriving from [redacted] 102 and 107. None of the 25 [redacted] progeny from [redacted] 101 share any of the introgression block found in [redacted]244. This molecular evidence along with the suggestion that both 228 and 244 have introgression blocks on homologous SBI-01 chromosomes strongly supports the hypothesis that inheritance of this introgression block was not random. It appears that strong selection was operating to transmit portions of this introgression block to apparently all BC_2F_1 progeny in these two families.

[redacted] (2*n* = 20; 1.72% introgression) contains common introgression with [redacted] Seven of its 9 introgression AFLP markers are also detected in [redacted] Although not analyzed with GISH, this individual likely contains a recombinant introgression block homologous to a portion of one of the alien substitution chromosomes present in 209.

SUMMARY

Introgression breeding utilizing the tertiary gene pool species *S. macrospermum* has resulted in the recovery of [redacted] chromosome backcrosses that contain wild species introgression. [redacted] were successfully recovered using the female hybrid gamete in combination with embryo rescue. Chromosome numbers were high and sterility a problem; however, viable [redacted] seed was set under backcrossing on 20% of the [redacted] plants. It is unclear what proportion of [redacted] ndividuals were produced through sexual backcrossing versus parthenogenesis of 20 chromosome egg cells, but both likely occurred.

Molecular markers verified that [redacted] individuals contained *S. macrospermum* introgression and measurements were between 0 and 18.6%. Molecular cytogenetic techniques, FISH and GISH, revealed that the introgression in the [redacted] plants was of three types: alien substitution, alien addition, and alien introgression lines. Male-sterility was the only obvious phenotypic trait observed that is likely caused by the introgression DNA.

Family differences were apparent in this germplasm. [redacted] and its [redacted] progeny showed the highest levels of fertility compared with families 102 and 107. [redacted] from this family were the only examples of alien substitution and addition lines observed. It is unknown whether the mixed pedigree of [redacted] is the cause of the increased fertility but it is a reasonable hypothesis. The family may have possessed a mix of alleles that facilitated recovery of alien addition and substitution lines as well as buffered the deleterious effects of recovered introgression. Such a hypothesis would suggest that using a complex and highly heterozygous population in introgression breeding may maximize the amount of recovered introgression as well as reduce the associated fertility problems.

The germplasm produced by from this investigation confirm that introgression and recovery of recombinants is possible through wide hybridization in sorghum. The introgression described herein documents an approach to introgression in sorghum that may not be limited to the Sorghum species. In the case of *S. macrospermum*, the value will only be known if derivatives are characterized. Using this research as a starting point, the true value of *S. macrospermum* genetic diversity can be determined.

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628

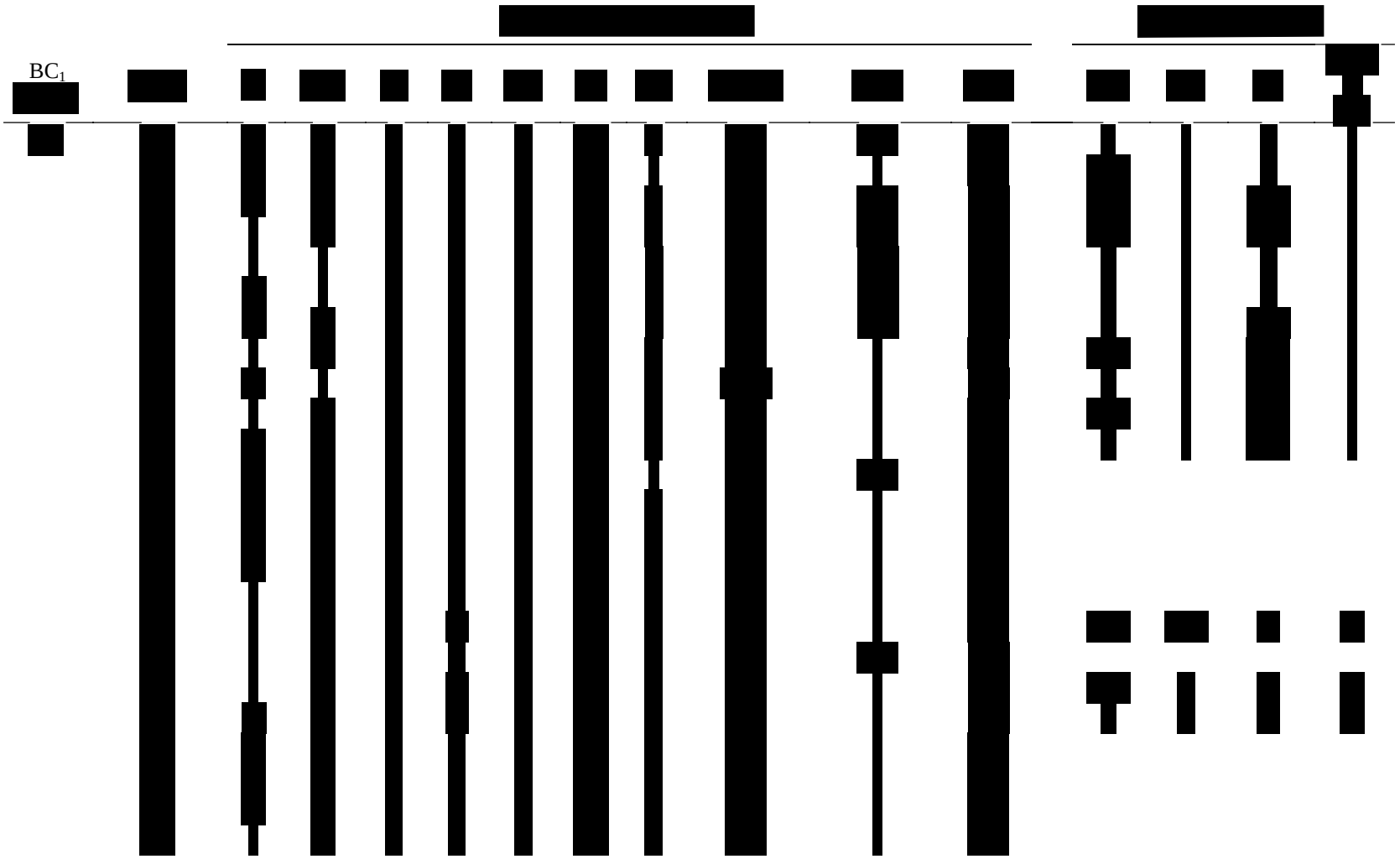
Table 1. Chromosome number and phenotypic data of [redacted] individuals ((*S. bicolor* x *S. macrospermum*) x *S. bicolor*) recovered using embryo rescue

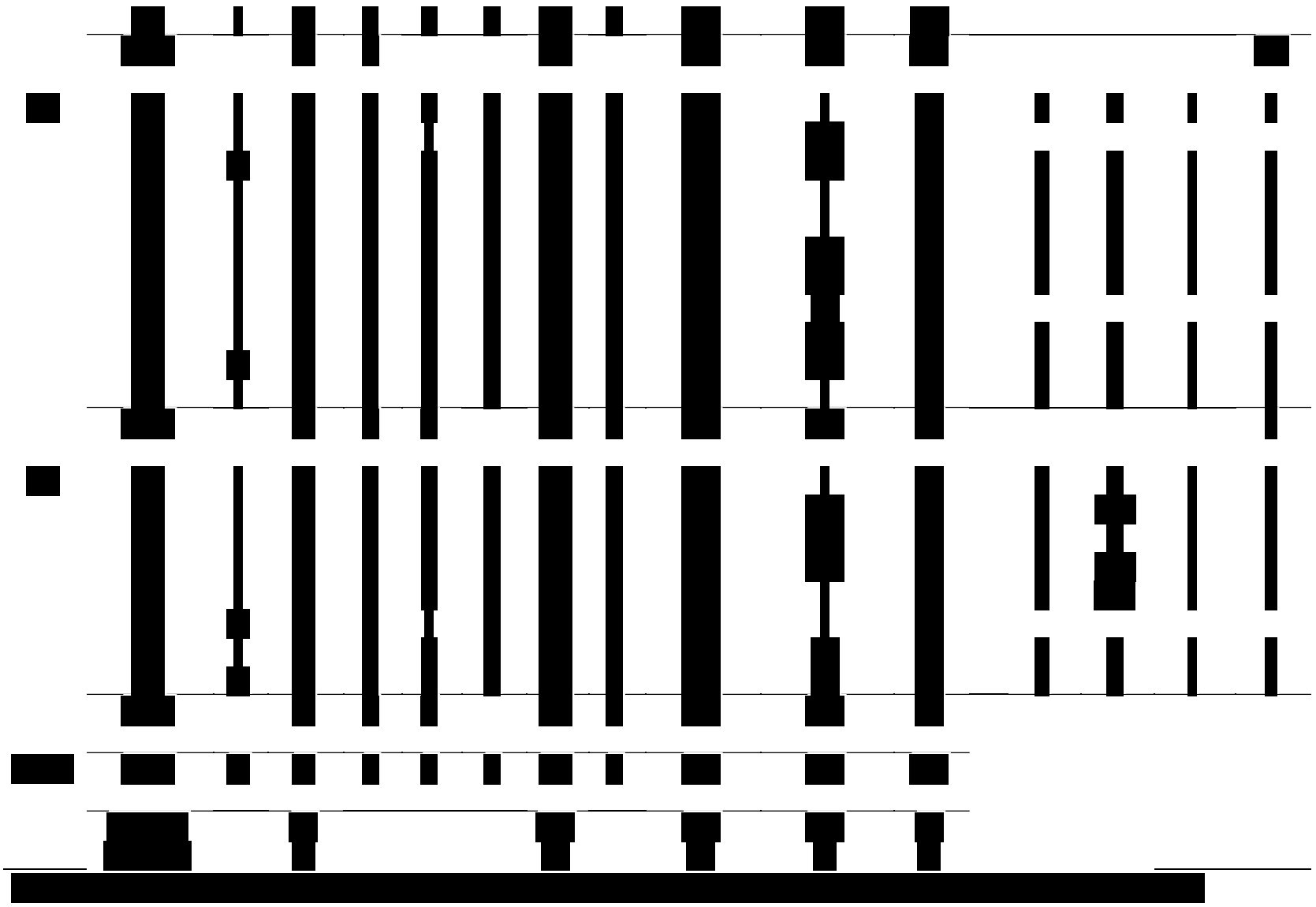
(p<.05)

629

630

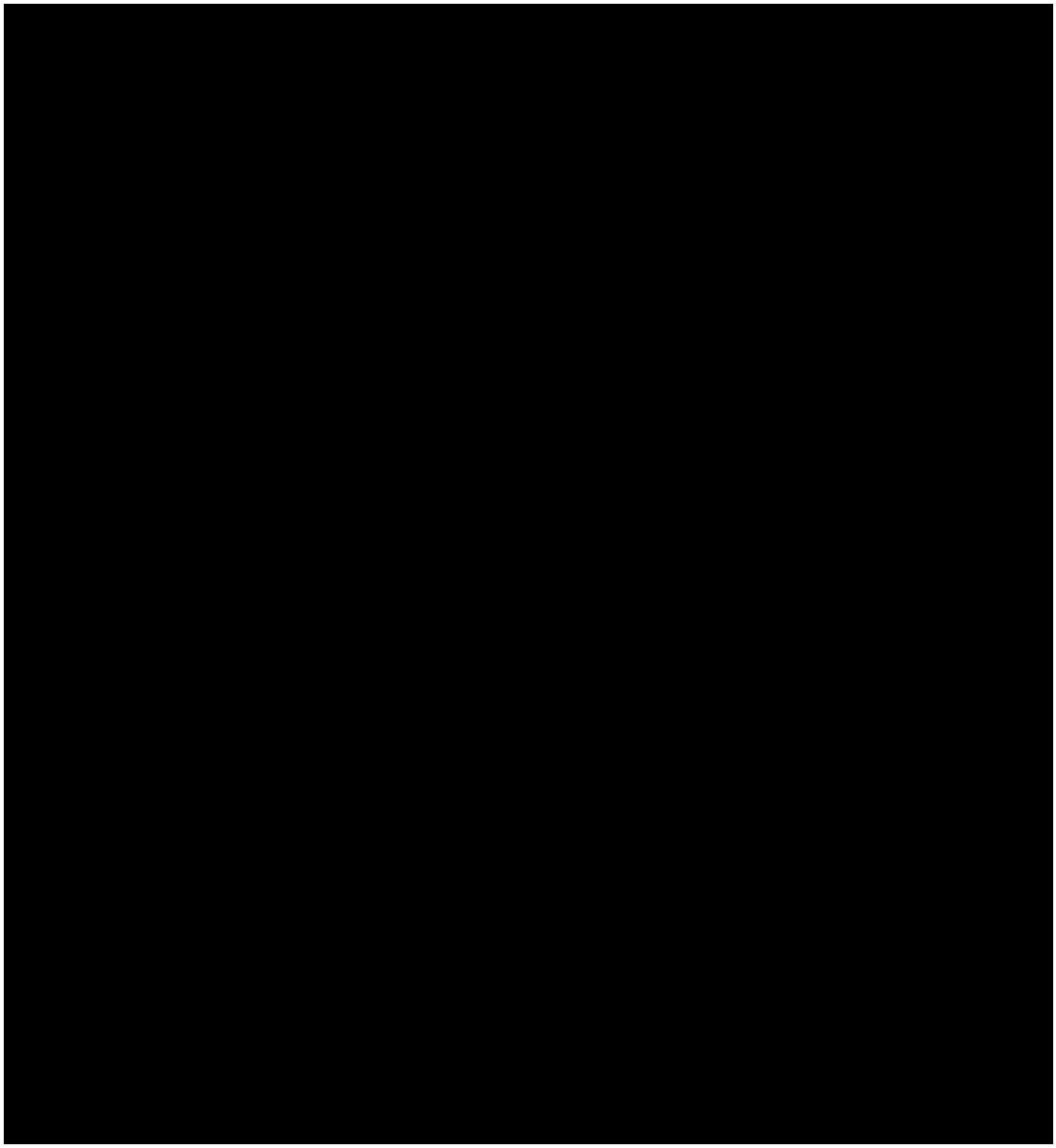
Table 2. Phenotypic data and *S. macrospermum* introgression estimates of BC_2F_1 individuals ((*S. bicolor* x *S. macrospermum*) x *S. bicolor*) and the recurrent parent. Phenotypic data for [redacted] progeny are given for some individuals.

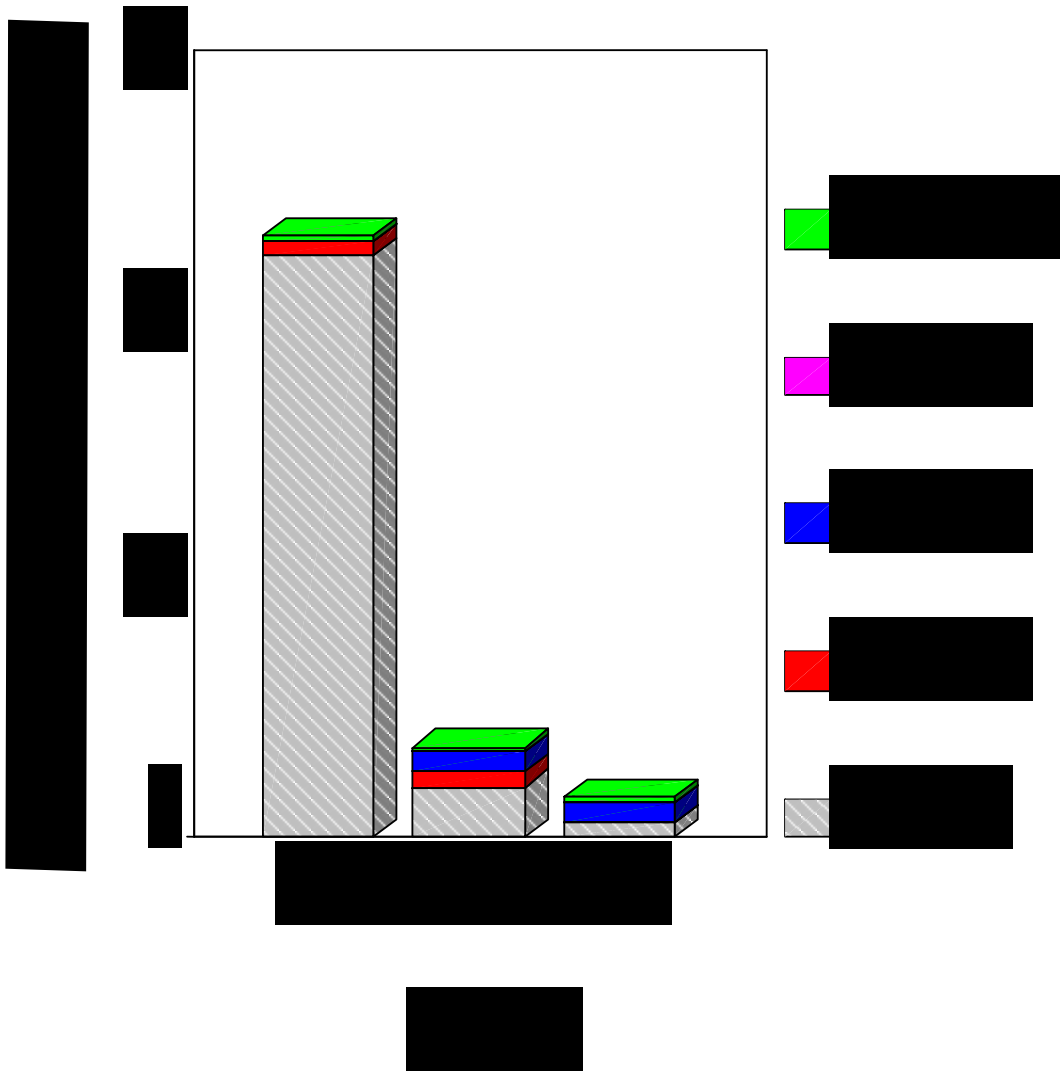


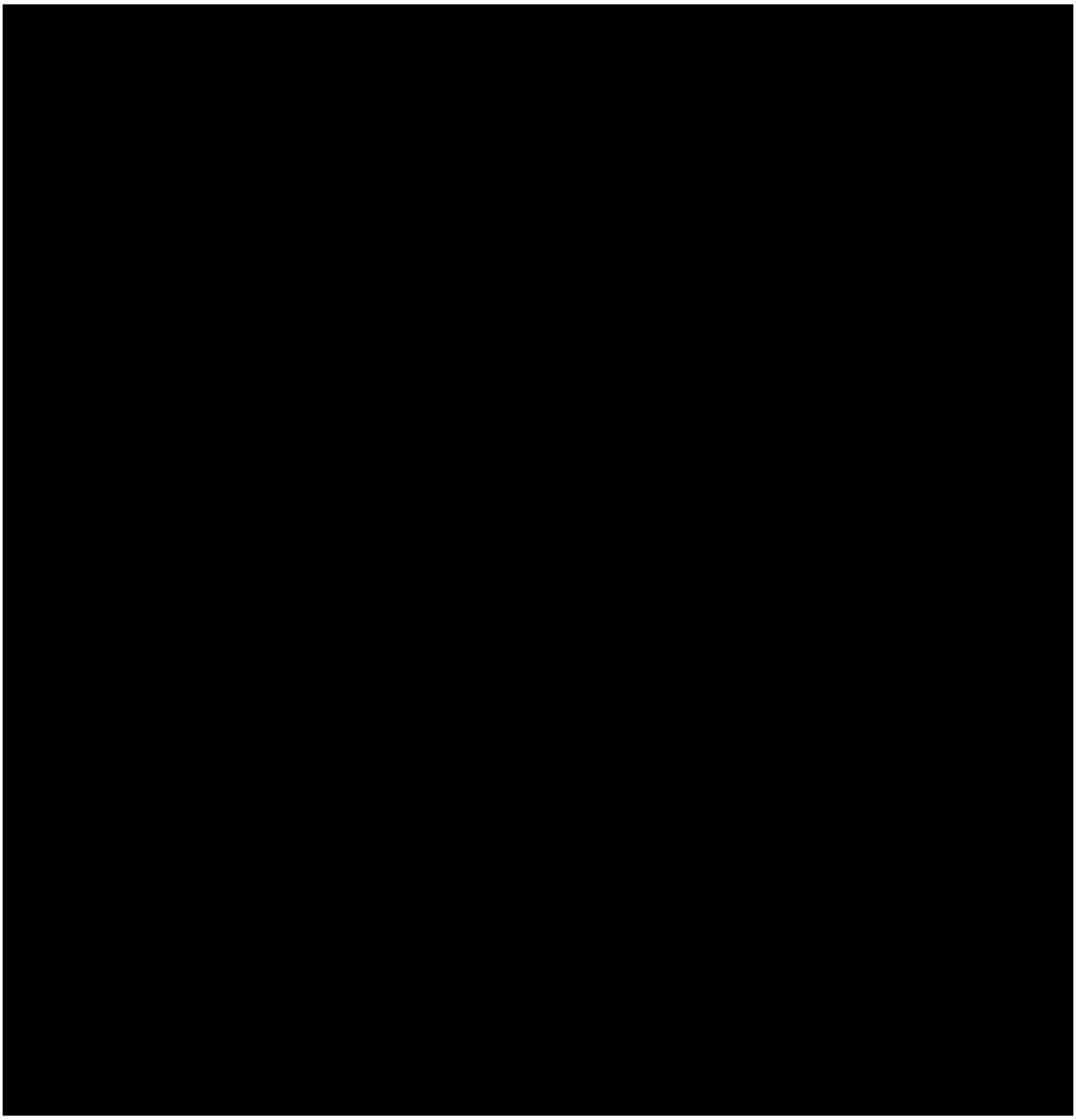


respectively

[redacted]







[redacted]