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BREEDING AND GENETICS

Hypoaneuploid Chromosome Substitution F_1 Hybrids of *Gossypium hirsutum* L. x *G. mustelinum* Miers ex Watt

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ABSTRACT

The infusion of new genetic diversity from related species into domesticated types of cotton (*Gossypium hirsutum* L.) will greatly increase opportunities for genetic improvement. We report here the development of the aneuploid F_1 chromosome substitution stocks in *G. hirsutum* for whole chromosomes and chromosome arms of *G. mustelinum* Miers ex Watt. These hypoaneuploid interspecific chromosome substitution stocks are an additional genetic resource for localization of genomic sequences, marker development, definition of linkage groups, and validation of genome maps. Hypoaneuploid plants that lack specific chromosomes or arms were detected by analysis of phenotypic syndromes and conventional meiotic metaphase-I configuration analysis of acetocarmine-stained microsporocytes ("pollen mother cells"), as well as by deletion analysis with chromosome specific SSR markers. Here, we report the development of 25 such hypoaneuploid hybrids, including 13 monosomic hybrids, each missing a different *G. hirsutum* chromosome (chromosome 1, 2, 4, 6, 7, 9, 10, 12, 16, 17, 18, 20, and 25, respectively), and 12 monotelodisomic (acrocentric) hybrids (Te05Lo, Te08Lo, Te11Lo, Te11sh, Te12Lo, Te14Lo, Te15Lo, Te20Lo, Te20sh, Te22Lo, Te22sh, and Te26sh) that are deficient for the respective distal segment of opposing *G. hirsutum* arms 5sh, 8sh, 11sh, 11Lo, 12sh, 14sh, 15sh, 20sh, 20Lo, 22sh, 22Lo, and 26Lo. Each of the interspecific F_1s reported here is a major step toward a development of the respective backcross disomic substitution line. Such lines are individually and collectively powerful resources for targeted germplasm introgression, genetic dissection, and genetic improvement of complex traits.

Major impediments to genetic improvement of cotton **(***Gossypium hirsutum* L., 2n = 52, $2(AD)_1$ genomic formula**)** include (1) the low genetic diversity of elite germplasm, (2) insufficient information about genes and gene interactions that control important traits, and (3) lack of suitable high-throughput methods for marker-assisted characterization and selection of intermediate generations during introgressive breeding.

The narrow germplasm base of the cotton breeding gene pool is a serious challenge to cotton improvement worldwide. Genetic diversity is quite low in *G. hirsutum*, and even more so among elite domesticated forms, in which it is deemed a significant factor that contributes to cotton yield stagnation, declining fiber quality, as well as to increasing genetic vulnerability to biotic and abiotic stresses. The need to diversify Upland cotton germplasm has long been recognized (USDA-NASS, 2002), and recent assessments (Bowman and Gutierrez, 2003; Bowman et al., 2003; Paterson et al., 2004) indicate that the germplasm pool of Upland cotton (*G. hirsutum*) in the US has been eroded seriously by excessive exploitation of a few elite lines over time. With reduced genetic diversity, and thus a smaller buffer against potentially adverse situations, the vulnerability of cotton to biotic and abiotic challenges is exacerbated, and there are fewer opportunities for significant genetic advances. To increase diversity, novel alleles associated with agronomic traits, fiber quality, and pest resistance must be discovered; furthermore, they must be bred into germplasm that is useful for cotton improvement.

Exotic species have contributed beneficial alleles for improving valuable traits in many plant species

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(Tanksley and McCouch, 1997). Wheat and potato, both polyploids with extensively integrated cytogenetic/breeding histories, are excellent examples (Friebe et al., 1991; Jansky, 2006; Knott, 1987). In the past, the most tangible derivatives from interspecific introgression were dominant alien genes that confer resistance or tolerance to disease and pests. Several analogous contributions to Upland cotton improvement also have been achieved, e.g., with bacterial blight and other traits affecting disease and pest resistance (Meyer and Meredith, 1978; Wallace and El-Zik, 1989; Wang et al., 2008). The advent of high-throughput genomics promises to extensively diversify the nature and increase visibility of the benefits from germplasm introgression (Fridman et al., 2000; Hyten et al., 2008; Mardis, 2008; Van der Hoeven et al., 2002). Similarly significant contributions to Upland cotton improvement are quite feasible.

The prospect of widening the genetic base of Upland cotton by incorporating genetic diversity from the wild tetraploid species has long been recognized (Davis, 1979; Kohel et al., 1977; USDA-NASS, 1974). Although alien germplasm represents a known source of unique alleles and perhaps novel genes (e.g., in rapidly evolving gene families), few agriculturally significant successes have actually resulted from interspecific introgression into cotton (Waghmare et al., 2005). The need to increase genetic variability of Upland cotton remains great. The most accessible genetic resources for interspecific introgression into Upland cotton are the other four species from its primary gene pool, i.e., *G. barbadense* L., *G. tomentosum* Nutt. ex Seem., *G. mustelinum* Miers ex Watt, and *G. darwinii* G. Watt, with which *G. hirsutum* forms reasonably fertile F_1 hybrids. All five of these species have 26-chromosome haploid genomes with grossly similar AD genomic architecture.

Most breeding efforts aimed at genome-wide introgression of AD-species germplasm into *G. hirsutum* have targeted *G. barbadense* (i.e., Pima or Egyptian cotton), because it, too, is cultivated and is known for superior fiber length and quality, both of which suggest it would be a better source of beneficial genes. The other AD-species are not domesticated or cultivated, and possess far less and shorter fiber than *G. hirsutum* and *G. barbadense*. However, comparative genomic marker and gene sequence data clearly show that *G. barbadense*, *G. tomentosum*, and *G. mustelinum* offer unique opportunities for genetic diversification of Upland cotton (Hulse et al., 2013; Lacape et al., 2007; Liu et al., 2000; Saha and Zipf, 1997).

Several factors impede derivation of useful genetic products from AD-genome introgression. Genetic incompatibilities between the AD-genome species are widespread at the whole-genome level. These are especially problematic because they tend to reduce transmission of much of the alien germplasm during backcrossing, and they undermine the performance of most products containing significant amounts of alien germplasm (Endrizzi et al., 1985). In addition, linkages between undesirable and desirable genes predispose potentially valuable introgression products containing the latter to failure. To circumvent such constraints, three possible approaches are (1) blind development of backcross-inbred lines (BILs) (Wehrhahn and Allard 1965), (2) the use of cytogenetic stocks to breed chromosome substitution (CS) lines (Dubcovsky et al., 1995; Joppa, 1993; Sears 1954), and (3) the use of marker-assisted selection to breed chromosome-segment introgression lines (ILs) (Eshed and Zamir, 1995; Wang et al., 2012). The latter two methods enable targeting of specific genomic regions.

Chromosome substitution lines can be developed using cytogenetic and/or marker-based methods (Fridman et al., 2000; Nadeau et al., 2000; Singer et al. 2004). Marker-based approaches require adequate numbers and distributions of mapped loci, and are more difficult to execute when the numbers of chromosomes and/or rates of recombination are high, as in cotton (2n = 52, ~4500 cM). Cytogenetic approaches require hypoaneuploid plants of the recurrent parental species, e.g., nullisomic, monosomic, or deletion stocks, each missing one or both copies of a specific chromosome or chromosome segment. The challenges in developing such aneuploid plants vary among different crop species and chromosomes. In cotton, nullisomic zygotes are not viable, so the procedures rely on monosomic rather than nullisomic types of *G. hirsutum* as the recurrent parents for hybridization and subsequent backcrossing, (Endrizzi et al., 1985; Saha et al., 2012; Stelly et al., 2005). Hypoaneuploids have been identified for about 75% coverage of the *G. hirsutum* genome and used as the donor parent to create near-isogenic hypoaneuploids of *G. hirsutum* TM-1 (Raska et al., 2005; Stelly, 1993; Stelly et al., 2005). For most Upland cotton hypoaneuploids, interspecific hypoaneuploid hybrids can be recovered if sufficient numbers of backcross progeny are screened, but the rates of sexual transmission via the seed parent vary widely among the aneuploids (Endrizzi et al., 1985). Using the hypoaneuploids of TM-1 as the recurrent backcross parents, 17 backcross-derived

G. barbadense chromosome substitution lines of Upland cotton already have been created and released (Stelly et al., 2005). We subsequently used the CS-B lines to identify chromosomal locations of important traits and detect cryptic beneficial genes and interactions (Saha et al., 2004, 2006, 2008, 2010, 2011, 2012). We also demonstrated that many of these lines are useful genetic resources for improving Upland cultivars (Jenkins et al., 2006, 2007). These findings demonstrate that chromosome substitution is an effective means of introgressing interspecific germplasm into Upland cotton, and suggest the approach should be extended to other AD-genome species, such as *G. tomentosum* and *G. mustelinum*. We previously developed hypoaneuploid F_1 plants that are hemizygous for different chromosomes of the Hawaiian species *G. tomentosum* (Saha et al., 2006); the hemizygous F_1s were used to initiate backcrossing of various *G. tomentosum* chromosomes into the TM-1 background (unpublished data).

The species *G. mustelinum* is native to the semiarid regions of northeastern Brazil. This species was first discovered by George Gardner in 1938 and rediscovered in 1965 (Aranha et al., 1969; Barroso et al., 2006, 2010; Neves et al., 1965). Like Upland cotton, this species has 26 gametic chromosomes, exhibits disomic meiotic pairing, and has a genome size approximately 2.2 to 2.9 Gbp. Among the five extant AD-genome species in the polyploid cotton lineage, *G. mustelinum* represents the alternate branch of the most basal split (Waghmare et al., 2005). Its foliage has the highest concentrations of the heliocides H_1 and H_4 and moderately high levels of gossypol (Khan et al., 1999). Except for gossypol, foliar concentrations of terpenoid aldehydes in the lysigenous glands were highest in *G. mustelinum* relative to 30 species representing A, B, C, D, F, G, K, and AD genomic groups of *Gossypium* (Khan et al., 1999). Many of these allochemicals are potentially useful for improving host-plant resistance in Upland cotton.

Here, we report the development of 25 novel hypoaneuploid hybrids involving *G. mustelinum*, including 13 monosomic hybrids, each missing a different *G. hirsutum* chromosome, and 12 acrocentric hybrids, each missing a large portion a specific *G. hirsutum* chromosome arm. Traditionally, these acrocentric cotton chromosomes have been alluded to as telosomes, and individuals carrying one such abnormal chromosome and one normal homolog have been described as monotelodisomic. Given the hemizygosity of specific *G. mustelinum* chromosomes or segments in these aneuploid interspecific F_1 stocks, they are useful resources for direct localization of genomic markers and linkage groups, and the integration of molecular maps. They are useful also as parents for development of backcross substitution lines, experimental applications of which include high-resolution genetic dissection of complex traits and detection of epistatic interactions.

MATERIALS AND METHODS

Plant Materials. We created near-isogenic interspecific F_1 hypoaneuploids that were hemizygous for a specific chromosome or segment of the *G. mustelinum* genome by emasculating flowers of near-isogenic *G. hirsutum* hypoaneuploids and subsequently pollinating them with *G. mustelinum* grown in a glasshouse at College Station, Texas (Fig. 1). Seed were harvested, ginned, delinted, and germinated (moist paper at ~30 °C) in a boll-wise manner. Germinated seeds were individually sown in Jiffy peat pellets, labeled, grown for 21 d in a greenhouse, and then space-transplanted to an irrigated field on the Texas A&M University campus. Seedlings were sown 36 in (~1 m) apart, with 10 plants per row, and end hills were separated by approximately 1.5 m; rows were sown at 40-in (~1 m) spacing.

Figure 1. Greenhouse-grown potted plants of *G. mustelinum* temporarily set outside for crossing with *G. hirsutum* hypoaneuploids developed and maintained at the Texas A&M University campus.

The near-isogenic *G. hirsutum* hypoaneuploid stocks noted above included a number of chromosomal deficiencies that were induced or discovered in various genotypic backgrounds, and repeatedly backcrossed as female with *G. hirsutum* inbred line TM-1, recovered, and then backcrossed again, eventually resulting in BC_nF_1 plants, where n ranges from five to more than 10 for various aneuploids. The inbred TM-1

was derived from the commercial variety 'Deltapine 14' by self-pollinating over 40 generations; the line is considered to be the primary genetic standard for cotton genetics and cytogenetics, as well as the molecular standard for *G. hirsutum* (Kohel et al., 2001).

The interspecific F_1 hybrid progeny were screened on a family-unit basis to identify progeny inheriting the same chromosomal deficiency as the maternal parent; for each deficiency, the opposing paternal single-copy genes and sequences would be rendered hemizygous for the donor allele, rather than heterozygous (alleles of donor and Upland parents) or homozygous (Upland allele, only). The hypoaneuploids included monosomes that are deficient for one chromosome: chromosomes 1, 2, 4, 6, 7, 9, 10, 12, 16, 17, 18, 20, and 25; and monotelodisomes that are deficient for one distal chromosome segment, due to replacement of one chromosome by a telosome (see below): Te01Lo, Te01sh, Te02Lo, Te02sh, Te03Lo, Te03sh, Te04Lo, Te04sh, Te05Lo, Te06Lo, Te06sh, Te07Lo, Te07sh, Te08Lo, Te09Lo, Te10Lo, Te10sh, Te11Lo, Te12Lo, Te14Lo, Te15Lo, Te16Lo, Te17sh, Te18sh, Te18Lo, Te20Lo, Te20sh, Te22Lo, Te22sh, Te25Lo, or Te26sh. Metaphase-I preparations from monosomic (2n = 51) and monotelodisomic (2n = 52) microsporocytes exhibited 25 II + I and 25 II + Ii or 25 II + slightly unequal II or 25 II + 2 I, respectively. "Telosome" and "monotelodisome" are historically accepted terms for aberrant chromosomes, but have been rendered obsolete by the terms "acrocentric" and "subacrocentric" chromosomes, which recognize that centromeres do not exist at the ends of chromosomes. Moreover, the term telosome now refers to telomere-associated high molecular weight complexes (Wright et al., 1992). In contemporary terms, each telosome relates to the presence of a derived acrocentric chromosome in place of a normal chromosome; monotelodisomic individuals have one normal chromosome and one acrocentric chromosome.

Each substitution monosomic F_1 plant (2n = 51) contains a full haploid complement (n = 26) from *G. mustelinum*, but lacks one chromosome of the normal *G. hirsutum* haploid complement, rendering the opposing *G. mustelinum* chromosome hemizygous. The same principles extend to the acrocentric (monotelodisomic) F_1 plants, but are complicated by the segmental nature of the deficiency and aneuploid nomenclature. Each monotelodisomic F_1 plant is expected to lack the distal end of one chromosome arm from the *G. hirsutum* seed parent. For example, F_1 hybrids containing the long-arm telosome of *G. hirsutum* chromosome-1 (Te01Lo) are expected to be hemizygous for the distal segment of the opposite (short) arm, i.e., arm 1sh. Interspecific Te01Lo hybrids would be disomic and heterozygous for parental differences in the long arm, but hemizygous for all *G. mustelinum* alleles opposite the maternal (Upland) short-arm segmental deficiency. Major features of *G. hirsutum* monosomic and monotelodisomic plants, including their cytogenetic behavior, transmission, and inheritance have been described elsewhere (Endrizzi et al., 1985). Most of the hypoaneuploid conditions elicit more or less unique phenotypic syndromes compared to the euploid parent, and are transmitted with good fidelity from the maternal parent, occurring in 10 to 50% of progenies.

Each of the reported hypoaneuploids was recovered as a segregate among progeny following interspecific pollination of the respective *G. hirsutum* hypoaneuploid. In parallel with the pedigree, the desired segregates were tentatively identified by plant phenotype. However, some related monotelodisomic and monosomic conditions lead to similar phenotypic syndromes. Moreover, misdivision of the univalent occasionally results in formation of monotelodisomic progeny by cotton monosomic seed parents, as in wheat (Sears, 1952), and meiotic numerical nondisjunction in monotelodisomic plants can lead to formation of monosomic progeny, e.g., H01 from Te01sh. Thus, we confirmed the cytogenetic constitution of the prospective segregates by meiotic metaphase-I configuration analysis of acetocarmine-stained microsporocytes ("pollen mother cells") as described by Endrizzi and Ramsay (1980).

Individuals identified or tentatively as hypoaneuploid at the Texas location were air-layered for vegetative increase. One or more clonal copies of each type were shipped to the Mississippi location.

Molecular Methods. Genomic DNA was extracted from young leaf samples of cytologically identified individual hypoaneuploid chromosome substitution F_1 hybrid plants at the Mississippi location using a QIAGEN DNeasy Plant Maxi kit (QIAGEN Inc, Valencia CA) and/or with a QIAGEN DNeasy Plant Mini kit. DNA solutions were diluted to a working concentration of 10 ng μl^{-1} and stored at 4 °C until PCR amplification. PCR reactions were performed in 10 µl volumes containing 10 ng of cotton template DNA, 1x GeneAmp PCR Gold buffer from Applied Biosystems (10x, 150 mM Tris-HCl, pH 8.0, 500 mM KCl), 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 µM of each primer, 0.35 µl of AmpliTaq Gold (Applied Biosystems, now Life Technologies, Grand Island, NY). Dye-labeled

chromosome specific forward and unlabeled reverse primers were used in all PCR reactions. We selected on an average five chromosome-specific SSR markers per hypoaneuploid line based on our previous reports (Gutierrez et al., 2009; Yu et al., 2011). The PCR amplification profile consisted of an initial denaturation of DNA at 94 °C for 5 min, followed by 35 cycles of 94 °C (step 1) for 30 sec, 60 °C (step 2) for 1 min, and 72 °C (step 3) for 1 min. After 35 cycles, the extension temperature of 72 °C was held for 8 min. PCR products of the labeled primers were diluted 20 to 50 times, depending on the concentration of the amplified products, for capillary electrophoresis following the overall manufacturer's protocol using ABI Prism 3100 DNA Analyzer (Applied Biosystems).

Slightly different procedures were followed for plants at the Texas location. Unlabeled, previously mapped cotton SSR primers were purchased from Integrated DNA Technologies, Coralville, Iowa. The PCR mixture (10 µl) used for SSR amplification contained 10 ng of template DNA, 0.4 µM of each SSR primer (forward and reverse), 0.1 mM of dNTPs (Qiagen), 0.4 U of Taq polymerase (GenScript, Piscataway, NJ), and 1 X PCR buffer, made from 10X buffer that included 1.5 mM $MgCl_2$. DNA was amplified for 38 cycles, each including 94 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 1 min, with initial denaturing of template DNA at 94 °C for 5 min and a final extension at 72 °C for 7 min. Amplified DNA samples were diluted by adding 5 µl of dilution buffer (Qiagen) and analyzed by an eGene capillary electrophoresis using a QIAxcel DNA High Resolution Kit (1200) (Qiagen). Bands were scored visually from images rendered by the machine software.

RESULTS AND DISCUSSION

The hypoaneuploid F_1 plants were identified among segregating progeny (euploid vs. aneuploid) based on maternal cytogenetic type, F_1 plant phenotypes and meiotic metaphase-I configuration analyses (Table 1, Figs. 2-4). Plant growth of euploid and aneuploid F_1 plants was far more vigorous than TM-1; under irrigated field conditions, main stems of many end-hill interspecific F1 seedlings attained diameters of 10 to 14 cm and exceeded 2.5 m in height. TM-1 seeds germinated in Texas during the spring (~1 April) flowered and set seed during the summer, whereas the donor species *G. mustelinum* had a significant juvenile period, after which plants typically flowered under winter greenhouse conditions at College Station; in several years, they sometimes set self seed. Some of the interspecific F_1 seedlings flowered in early fall, and most set seeds in during the winter under heated greenhouse conditions; under field conditions, some began floral bud development but none flowered or set seed prior to implementation of the local plant destruction deadlines set by the statewide Boll Weevil Eradication program in College Station, TX.

Figure 2. Various parts of a hypoaneuploid F_1 plant (flower, boll with fiber, mature green boll, flower bud).

Figure 3. A greenhouse-grown hypoaneuploid F_1 plant missing *G. hirsutum* chromosome 25.

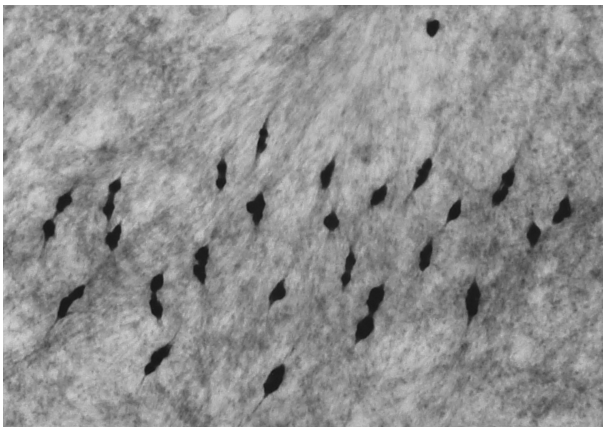

Figure 4. Cytological detection of monosomy by analysis of chromosome configurations in a microsporocyte metaphase-I cell. The image shows 25 bivalents and 1 univalent (near top margin) and, thus, a total of 2n = 51 chromosomes. The cell was sampled from an individual backcross progeny from a maternal parent also monosomic for chromosome 4. The similarity of chromosome number and phenotypic syndromes (not shown) indicated that this progeny plant was also monosomic for chromosome 4.

Table 1. Monosomic and monotelodisomic *G. hirsutum* x *G. mustelinum* interspecific F_1 hybrid chromosome substitution stocks deficient for specific *G. hirsutum* chromosomes and chromosome arms.

Original Plant Identity	Cytological Configuration[z]	Designation	Chromosomal Deficiency	Phenotypic Syndrome
200301511.06	25 II + large I	CS-M-H01-F1	1	Smaller plant, small narrow leaf, narrow or twisted bracts, small oblong boll
200301512.06	25 II + large I	CS-M-H02-F1	2	Smaller plant, smaller leaf with cupped margins, shorter sympodia, smaller round bolls with mid-locule furrow
200301514.10	25 II + large I	CS-M-H04-F1	4	Bushy plant, wavy margins near leaf base, long peduncle and boll
200301515.04	25 II + very large I	CS-M-H06-F1	6	Slower plant growth, reduced branching and clustered sympodia and small bolls
200301526.02	25 II + large I	CS-M-H07-F1	7	Light green plant with large crinkled leaf with secondary lobing; contorted bracteole and calyx; toothy bracteole, shorter sympodia.
200401472.05	25 II + large I	CS-M-H09-F1	9	Slower growing and rigid plant. Pitted boll.
200301533.12	25 II + very large I	CS-M-H10-F1	10	Slower plant with fewer branches. Large, round flat leaf. Clustered sympodia. Large abnormal stigma. Larger seed.
200401512.09	25 II + large I	CS-M-H11-F1	11	Compact bushy plant. Dark and glossy leaf. Small flower and boll. Deep mid-locule furrow.
200301542.14	25 II + large I	CS-M-H12-F1	12	Smaller, narrower and crinkled leaf. Larger flower with petals more open. Semi-sterile (poor pollen shed).
200401544.09	25 II + small I	CS-M-H16-F1	16	Lighter green plant. Crinkled leaf. Shorter sympodia. Smaller boll.
200301565.20	25 II + small I	CS-M-H17-F1	17	Smaller plant. Smaller, narrower and more pointed leaf. Longer style and stigma. Seed usually larger.
200301572.12	25 II + small I	CS-M-H18-F1	18	Smaller plant. Smaller, narrower and more pointed leaf. Longer peduncle.
200401566.01	25 II + medium I	CS-M-H25-F1	25	Slower plant. Smaller leaf. Clustered sympodia.
200301611.10	25 II + large Ii	CS-M-Te05Lo-F1	5, short arm	Narrow leaf with shallow base. Narrow bracteole.
200301614.08	25 II + v. large II slightly unequal	CS-M-Te08Lo-F1	8, short arm	Small dark green plant. Smaller leaf and flower. Small boll with pointed tip.
200301622.10	25 II + v. large II slightly unequal	CS-M-Te11Lo-F1	11, short arm	Slower, bushy plant. Darker and glossy leaf. Smaller flower. Short bracteole. Smaller boll with mid-locule furrow.
200301625.14	25 II + v. large Ii	CS-M-Te11sh-F1	11, long arm	Compact bushy plant. Dark and glossy leaf. Small flower and boll. Deep mid-locule furrow.
200301643.08	25 II + large Ii	CS-M-Te12Lo-F1	12, short arm	Larger leaf. Narrow bracteole. Longer style and stigma.
200301645.10	25 II + medium-small slightly unequal II	CS-M-Te14Lo-F1	14, short arm	Most plants slower. Slightly smaller leaf. Semi-short sympodia. Mid-locule furrow.
200301653.16	25 II + small slightly unequal II	CS-M-Te15Lo-F1	15, short arm	Narrow, twisted bracteole.
200301661.18	25 II + small slightly unequal II	CS-M-Te20Lo-F1	20, short arm	Reduced branching. Larger and usually darker leaf. Semi-short sympodia. Larger but shorter stigma.
200401574.13	25 II + small Ii	CS-M-Te20sh-F1	20, long arm	Reduced branching. Larger darker leaf. Clustered sympodia.
200301675.16	25 II + medium slightly unequal II (often 25 II + 2 I)	CS-M-Te22Lo-F1	22, short arm	Lighter green, concave leaf. Long style and stigma. Long bracteole teeth and boll.
200301682.18	25 II + medium Ii	CS-M-Te22sh-F1	Long arm of 22	Bushy plant. Narrow bracteole. Longer peduncle.
200301722.04	25 II + small Ii	CS-M-Te26sh-F1	Long arm of 26	Smaller, narrower leaf. Short sympodia. Prolific flowering. Petals more open.

[z] **Configuration symbols: II = bivalent, I = univalent, Ii = unequal bivalent.**

The leaves, flowers, and bracts of the euploid and hypoaneuploid F_1 plants were larger than those of TM-1. Flowers of hybrid plants were intermediate in size, morphology, and pigmentation relative to the parents. Floral petals were more yellow in the F_1 hybrids than in TM-1, and exhibited a light petal spot at the base of

each petal. Mature anthers and pollen of F_1 plants were bright yellow (Fig. 2) and the hybrids had very dark green leaf color compared to TM-1. Seeds formed by the hybrids were covered with light-tan short and long fibers (Fig. 2), distinctly different in length and color from Upland cotton, hence essentially intermediate compared to both parental species.

Phenotypes of some aneuploid stocks were quite distinctive, in a few cases, unexpectedly so (Table 1). For example, leaves of plants hemizygous for chromosome 1 (CS-M01-F_1) were silvery gray in color, whereas those of plants missing chromosome 7 or 16 were lighter or brighter green. Whereas the sympodia of the TM-1 aneuploids missing chromosomes 6, 7, 10, or 25 are shorter than sympodia of TM-1, the differences in sympodium length among interspecific F_1 plants were relatively smaller.

The interspecific F_1 progeny families from the respective *G. hirsutum* hypoaneuploid maternal parent were grown, and the desired hypoaneuploids were tentatively identified by plant phenotype and then checked by metaphase-I analysis (Fig. 4, Table 1). Such analyses help guard against misdivision of the monosomic univalent and the inadvertent recovery of a related monotelodisomic, e.g., H10 giving rise to Te10sh, a phenomenon reported in wheat (Sears, 1952). The analyses also alert us to the derivation of centric fragment(s). Conversely, analyses of progeny from monotelodisomic female parents provided a check against inadvertently selecting a related or unrelated monosomic progeny, rather than monotelodisomic ones. Thus, we used meiotic metaphase I configuration analysis of acetocarmine-stained microsporocytes to help confirm sexual transmission of the intended cytogenetic deficiency (monosomy or monotelodisomy) to the selected progeny.

The phenotypic and cytogenetic selections were further confirmed by molecular marker analysis using chromosome-specific SSR markers following the deletion analysis methods (Guo et al., 2008; Gutierrez et al., 2009; Liu et al., 2000). On average, five chromosome-specific SSR primer pairs were used for individual missing chromosome or chromosome segment. To localize SSR loci to chromosomes, we screened the chromatin-deficient substitution stocks (BC_0F_1) for the TM-1 allele, using labeled (Fig. 5) and/or unlabeled (Fig. 6) primers. For SSR loci located at sites other than the chromatin deficient segment, the Upland marker would be present and the F_1 hybrid would exhibit heterozygous phenotype. In contrast, if an SSR locus were on the monosomic or monotelodisomic deficient segment of the interspecific hypoaneuploid chromosome substitution F_1 hybrid, the electropherogram would lack the TM-1 allele and exhibit hemizygous (homozygous-like) pattern for the donor (*G. mustelinum*) allele. The results of the deletion analysis are presented in Table 2, confirming the cytological results. Several SSR primer pairs yielded more than one marker per primer pair, which is expected considering the tetraploid nature of the genome. However, we confirmed the genetic identity of the plant according to absence of a chromosome-specific TM-1 allele and hemizygous presence of the corresponding donor allele from *G. mustelinum*.

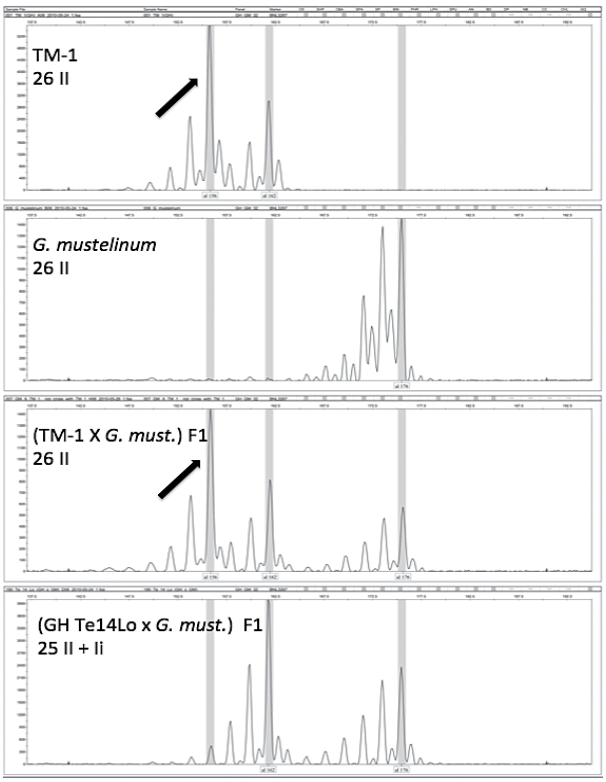

Figure 5. Loss-of-heterozygosity test to confirm the chromosomal identity of an interspecific CS-M F_1 segmental hypoaneuploid using SSRs and an ABI 3100. We detected amplicons after PCR with labeled primers for SSR marker BNL 3267, using DNAs from *G. hirsutum* TM-1 (top), *G. mustelinum* (next to top), their euploid F_1 (next to bottom) and a monotelodisomic (Te14Lo x *G. mustelinum*) F_1 plant with 25II + Ii meiotic configurations. BNL was mapped previously to chromosome arm 14-short (c14sh). Top: The *G. hirsutum* recurrent parent (TM-1) exhibits two sets of stuttered SSR bands, the largest being at 156 and 162 bp. Second from top: the germplasm donor, *G. mustelinum*, exhibits one set of strongly stuttered SSR bands, the largest at 172 bp; these could represent amplicons from one locus, or two loci with similarly sized amplicons. More importantly, the normal F_1 hybrid has bands of both parents, whereas the Te14Lo monotelodisomic F_1 hybrid lacks the 156-bp major band from *G. hirsutum*, supporting the inference that it is deficient of the *G. hirsutum* arm 14sh.

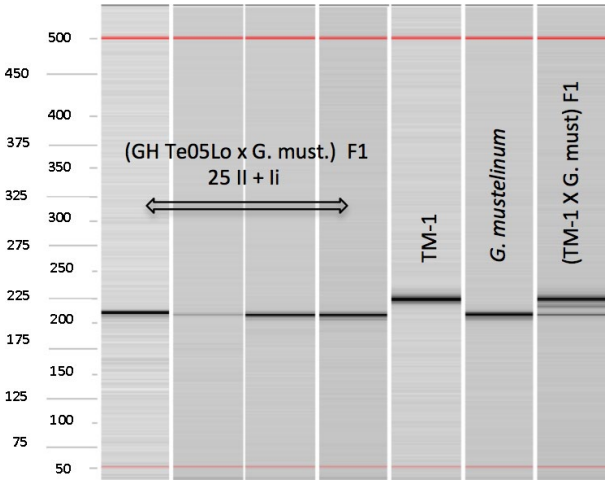

Figure 6. Detection of hemizygosity in a CS-M segmental aneuploid for the SSR marker BNL 3995 previously mapped to chromosome arm 5-short (c05sh). Single SSR bands are shown for *G. hirsutum* TM-1 (recurrent parent) and the germplasm donor, *G. mustelinum*. The normal F_1 hybrid has bands of both parents, whereas the TM-1 band is missing from each of several putative Te5Lo monotelodisomic F_1 hybrids, supporting the inference that each is deficient of the *G. hirsutum* arm 5sh. The images depict SSR amplicons and are generated automatically by software of an eGene nuclei acid fragment analyzer, based on the luminosity values (darkness) across time (top to bottom) at a fixed point along the electrophoresis path of each capillary.

The recovery of interspecific F_1 hypoaneuploids represents the first step of the interspecific breeding process that leads to substitution (i.e., chromosome-specific introgression of germplasm from an alien genome). In addition, these first-generation F_1 hybrid cytogenetic stocks are powerful analytical tools for physical mapping, dosage analysis, genetic marker localization, and validation of genome maps. Most of the published cotton genomic maps exhibit one or more of the following limitations: the number of linkage groups does not equal the haploid chromosome number, many loci remain unlinked, chromosomal identifications are incomplete, orientations are incomplete or absent, and multiple nomenclatures exist for linkage groups (Han et al., 2004; Nguyen et al., 2004; Reinisch et al., 1994). Interspecific F_1 hypoaneuploid stocks can help overcome some of these limitations, because all loci in euploid chromosomes exhibit high levels of heterozygosity (polymorphism), and all loci spanned by the chromosomal or segmental deficiency exhibit hemizygosity and loss of heterozygosity. Thus, previously determined chromosomal associations can be further confirmed (e.g., for linkage groups and individual markers). These cytogenetic deficient lines will help to verify chromosomal associations of markers and linkage groups, assign loci or molecular markers to a specific chromosome or chromosome segment, and detect synteny between loosely linked linkage groups and/or loci (Gao et al., 2004). Moreover, by integrating laboratory and population-specific maps, they can establish a biologically based, common nomenclature for chromosomes and linkage groups (Kim et al., 2005). Several complementary physical mapping methods are available for cotton genomics (Gao et al., 2004; Hanson et al., 1995; Wang et al., 2006; Zhang et al., 2012), including the deficiency analysis of hypoaneuploid interspecific F_1 hybrids (Guo et al., 2008; Gutierrez et al., 2009; Liu et al., 2000).

These hypoaneuploid stocks will help to validate and integrate map information from different labs into a consensus map that will benefit the entire cotton research community. Until now, only the aneuploid series of *G. barbadense* and *G. tomentosum* were available for development of chromosome-specific markers (Gutierrez et al., 2009; Han et al., 2004; Liu et al., 2000). Relative to *G. barbadense* and *G. tomentosum*, these new aneuploid lines from *G. mustelinum* provide additional sources for polymorphisms with *G. hirsutum*; thus, this new series of *G. mustelinum* substitution aneuploids will permit chromosomal localization of additional molecular marker loci, including those uniquely applicable to introgression of *G. mustelinum* germplasm (Hulse et al., 2013; unpublished data).

G. mustelinum is phenotypically distinct from *G. hirsutum* in its fruit and seed characteristics (Pickersgill et al., 1975); it also possesses many allochemicals for resistance to many cotton insects (Khan et al., 1999). These aneuploid substitution stocks provide the means to launch backcross chromosome substitution line development, which is critical to successful wide-cross alien gene transfer for improved agronomic performance, fiber traits including yield, and resistance traits against biotic and abiotic factors. Many of these traits are genetically complex and difficult to improve through wide-cross introgression using conventional breeding methods (Van Esbroeck and Bowman, 1998). Our previous studies with *G. barbadense* chromosome substitution lines (CS-B lines) have demonstrated that chromosome-specific introgression using chromosome substitution lines complement conventional methods of interspecific introgression and unveil many cryptic beneficial alleles useful for Upland cotton improvement (Jenkins et al., 2006, 2007;

Saha et al., 2010). Alien germplasm utilization seems more likely to be successful when retention of the alien germplasm is forced, as by hemizygosity during backcrossing. Thus, we expect to initially use the *G. mustelinum* F_1 hypoaneuploids as parents for developing backcross substitution lines, and then to develop chromosome-specific interspecific RILs that enable high-resolution QTL mapping, as is now underway for *G. barbadense* germplasm (Saha et al., 2012, 2013; Stelly et al., 2005).

Table 2. Molecular confirmation of the aneuploid BC_0F_1 plants.

Plant New ID (CS-M Series)	SSR Primer	TM-1 SSR Marker bp	*G. mustelinum* SSR Marker bp	TM-1 x *G. mustelinum* BC_0F_1 Aneuploid SSR Marker bp	Chromosome or Arm Location
200408110.08	BNL1667	164[z]	147, 188, 190	147, 188, 190	1
200408110.08	BNL1693	241[z]	245, 247, 258, 260	245, 247, 258, 260	1
200408110.08	BNL2664	125[z]	123	123	1
200408110.10	BNL1897	128, 145[z]	128, 155	128, 155	2
200408110.10	BNL3413	179[z]	158	120, 158	2
200408110.10	BNL3545-2[zz]	115, 139, 189[z]	129, 179	115, 129, 139, 179	2
200408110.10	BNL3971	145[z]	154	143, 154	2
200408110.10	CIR381-2	245, 265[z]	243, 263	243, 245, 263	2
200408111.01	BNL530	140[z]	136	136, 138	4
200408111.01	BNL2572	253[z], 331	266, 268, 270, 333	266, 268, 270, 331, 333	4
200408111.01	BNL3433	232[z]	236	218, 236	4
200408111.01	BNL3988	143[z], 145[z]	96, 115	96, 115	4
200408112.07	BNL3241	121[z], 329[z]	123, 334	123, 334	5sh
200408112.07	BNL3995	196[z]	181	181	5sh
200408111.02	BNL2569	79, 170[z]	79, 164	79, 164	6
200408111.02	BNL2884	154[z], 165[z]	179	179	6
200408111.02	BNL3359	208[z], 223	217	217, 223	6
200408111.02	BNL3650	355[z]	347	347	6
200708058.06	BNL1694	223, 238[z]	223, 235	223, 235	7
200708058.06	BNL2634	197[z]	198, 262	198, 262	7
200708058.06	BNL3415	235[z]	255	255	7
200708058.06	BNL3793	157[z]	159	159	7
200708064.03	BNL387	211[z]	194, 206	194, 206, 212	8sh
200708064.03	BNL2772	181[z]	171	171, 178	8sh
200708064.03	BNL3556	122, 135[z]	122, 130	122, 130	8sh
200708059.08	BNL1414	137[z], 160	117, 125, 145	117, 125, 145, 160	9
200708059.08	BNL2608	183[z]	180, 185	180, 185	9
200708059.08	BNL2847	233[z]	222	222	9
200408111.06	BNL256	180, 200[z], 206[z]	180, 208, 214	180, 208, 214	10
200408111.06	BNL3790	164[z]	144	144	10
200408111.06	BNL3895	178, 188[z]	180, 190	178, 180, 190	10
200408111.06	BNL4102	233[z], 250[z]	257	257	10
200408111.09	BNL116	141[z], 143	132, 134	132, 134, 143	12
200408111.09	BNL391	307[z]	252, 259, 294	252, 259, 294	12
200408111.09	BNL1679	143, 162[z]	143, 166	143, 166	12
200701413.01	BNL3261	211[z]	217	217	12
200701413.01	BNL1673	180, 197[z]	174, 181	174, 181	12
200701413.01	BNL4059	225[z], 220,218	219	219	12
200408111.09	BNL2621	202[z], 209	194, 210	194, 209, 210	12
200408113.05	BNL3099	170[z]	163	163, 172	14sh
200408113.05	BNL3267	156[z], 162	176	162, 176	14sh
200408113.05	BNL3545-1[y]	115[z], 139[z], 189	129, 179	129, 179, 189	14sh

(continued next page)

Table 2. (continued)

200408113.05	BNL3644	187, 193[z]	185, 187, 194, 202	185, 187, 194, 202	14sh
200408113.05	CIR246	167[z]	156	156	14sh
200408113.05	CIR381-1	245[z], 265	243, 263	243, 263, 265	14sh
200408113.07	BNL2920	135[z]	132, 153	132, 153	15sh
200408113.07	BNL4023	277[z]	275	275	15sh
200408113.07	BNL4080	202[z]	198	198	15sh
200408113.07	BNL4082	171[z]	161, 163, 167	129, 161, 163, 167	15sh
200401544.09	BNL2734	230[z], 232[z]	211	211	16
200401544.09	BNL3008	130[z]	131, 133, 171, 173, 175	96, 129, 171, 173, 175	16
200708062.01	BNL3008	135[z]	176, 181	176, 181	16
200708062.01	BNL1395	154[z], 164	161, 174	161, 174	16
200708062.01	BNL1122	167[z], 177	175, 187	175, 187	16
200401544.09	BNL3065	190[z]	180	180	16
200401544.09	BNL3500	63, 81[z], 108	63, 105	63, 105, 108	16
200408112.02	BNL2471	196[z]	194	194	17
200408112.02	BNL2496A	98, 113[z]	98, 104	98, 104	17
200408112.02	BNL3371	169, 196[z]	166	166, 169	17
200408112.02	BNL4073	114[z], 116	122, 124	122, 124	17
200408112.03	BNL1721	190[z]	171	171	18
200408112.03	BNL4007	172[z]	168	168	18
200408112.03	BNL4029	245, 389[z]	384, 398	245, 384, 398	18
200401574.13	BNL946	326[z]	235, 298, 307, 341	265, 298, 307, 341	20Lo
200401574.13	BNL3379	196[z]	184	184	20Lo
200401574.13	BNL3660	133, 136, 138, 141, 143, 175[z]	133, 136, 138, 141, 145	133, 136, 138, 141, 143, 145	20Lo
200401574.13	BNL3948	97[z]	95	95	20Lo
200408113.08	BNL169	198[z]	194, 209	194, 209	20sh
200408113.08	BNL3071	152, 160[z]	152, 156	152, 156	20sh
200408113.08	BNL3646	156[z]	148	148	20sh
200408113.08	BNL3993	205[z]	217	217	20sh
200401574.13	BNL358	127[z]	169, 173, 274, 282, 296, 316	169, 173, 274, 282, 296, 316	22Lo
200401574.13	BNL3873	124[z]	120	120	22Lo
200401574.13	BNL3994	97, 124[z]	90, 101	90, 97, 101	22Lo
200408114.02	BNL358	127[z]	169, 173, 274, 282, 296, 316	169, 173, 274, 282, 296, 316	22Lo
200408114.02	BNL3873	124[z]	120	120	22Lo
200408114.02	BNL3994	97, 124[z]	90, 101	90, 97, 101	22Lo
200401566.01	BNL150	122[z], 167[z]	130, 175, 202	130, 175, 202	25
200908081.01	BNL3436	178, 196[z], 199	178, 193	178, 193	25
200908081.01	BNL3806	171, 207[z]	171, 200	171, 200	25
200908081.01	BNL1047	150, 160[z], 162	150	150	25
200908081.01	BNL3594	182, 190[z],197,205	184, 212,220	184, 212,220	25
200401566.01	BNL2691	217, 241[z], 257	257, 261	217, 257, 261	25
200401566.01	BNL3190	165[z]	154	154	25
200401566.01	BNL3806	172, 207[z]	172, 201	172, 201	25
200408114.06	BNL840	158[z]	160	158	26Lo
200408114.06	BNL1045	200[z], 213	199, 204, 212, 213, 218	199, 204, 212, 213, 218	26Lo
200408114.06	BNL2495	195[z]	187	187	26Lo
200408114.06	BNL3368	154, 165[z]	154, 177	154, 177	26Lo

[z] **Missing band in BC0F1 aneuploid, sh = Short arm, Lo = Long arm.**

[y] **Duplicated loci are denoted by a numerical suffix (-1, -2, or -3) that also denotes relative (increasing) *G. hirsutum* allele fragment size (Gutierrez et al., 2009).**

In conclusion, these new aneuploid chromosome substitution stocks are a useful resource for localization of genomic markers, for integration of molecular maps, and as the launch pad for developing backcrossed chromosome substitution lines, high-resolution chromosome-specific genetic dissection of complex traits.

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