DISSECTING QUANTITATIVE VARIATION INTROGRESSED INTO BERMUDAGRASS AND UPLAND COTTON

by

SAMEER KHANAL

(Under the Direction of Andrew H. Paterson)

ABSTRACT

Mendelizing quantitative trait loci (QTL) and identifying 'stable' QTL are prerequisites for marker-assisted selection in crop improvement. Here, we used molecular markers to study genetic basis of quantitative variation introgressed into bermudagrass and Upland cotton. Bermudagrass lags in genomic and molecular breeding resources. We screened 2,017 sugarcane primer pairs (PPs) for transferability and polymorphisms in bermudagrass germplasm. A subset of useful markers was used to build genetic linkage maps of two bermudagrass species, *Cynodon dactylon* (T89) and *Cynodon transvaalensis* (T574) and genetic architecture of one foliage (canopy height, HT), two stolon (stolon internode length, ILEN and length of the longest stolon LLS), and two leaf traits (leaf length, LLEN and leaf width, LW) in 110 F_1 individuals was reported. This early QTL study in bermudagrass adds to molecular breeding resources and information in the genus.

In Upland cotton, we developed QTL-stacked populations in genetic backgrounds of six elite *G. hirsutum* backgrounds targeting nine fiber QTL alleles introgressed into advanced-backcross *G. hirsutum* lines affecting fiber elongation (three each from *G. tomentosum* and *G. mustelinum*), fiber fineness (one from *G. tomentosum*), fiber strength (one from *G. mustelinum*), and fiber length (one from *G. barbadense*). A total of thirty-eight QTL-stacked F_1 were selfed to generate F_2 populations segregating at two to four QTL regions, which were field tested for fiber quality traits, genotyped at target QTL regions, selectively advanced to $F_{2:3}$ generation and assessed at two different locations (Athens and Tifton, GA). Favorable

shifts in average phenotypes of selected traits were partially explained by genotypes at introgressed QTL regions, while a subset of QTL and some digenic combinations was also consistent across backgrounds and/or between generations. Our results attest to the promise of MAS as a molecular breeding tool for simultaneously validating QTL effects and developing QTL-stacked germplasm in different genetic backgrounds. Resources developed in this study are valuable for molecular breeding applications in bermudagrass and Upland cotton.

INDEX WORDS: Genetic diversity, genetic linkage maps, quantitative trait loci, QTL pyramiding

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DEDICATION

I dedicated this dissertation to the memory of my parents Mrs. Jiba Manjari Sharma (Aama) and Mr. Chiranjeevee Khanal (Buwa), my girlfriend Miss Pratima Subedi (Sym/Kanchchu), and my sister-in-law Mrs. Reecha Pant Khanal.

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CHAPTER 1

INTRODUCTION

Bermudagrass in a Nutshell

Bermudagrass is a common name referring to several resilient perennial grasses of the genus *Cynodon* (L.) Rich., which typically colonize warmer climates and are widely used for turf in the tropical, subtropical, and warm temperate regions of the world. Common bermudagrass [*Cynodon dactylon* (L.) Pers.] was introduced into the United States in the mid-1700's and within decades, it naturalized in the warm regions of the country, becoming the most prominent pasture grass of the Southern states (Harlan 1970; Wu 2011). The United States National Plant Germplasm System (NPGS) records eight extant species and ten varieties, following the revised taxonomic classification by Harlan (1970a). The genus belongs to family Poaceae and subfamily Chloridoideae (Clayton and Renvoize 1986), in the "PACC" clade that contains subfamilies Panicoideae, Arundinoideae, Centothecoideae, and Chloridoideae (Kim et al 2009). While some Panicoids including maize, sorghum, and sugarcane have been extensively studied, Chloridoideae subfamily members lag in scientific exploration. Nonetheless, Chloridoideae is one of the most important subfamilies including a number of warm-season turfgrass species with the C4 photosynthetic pathway, such as bermudagrass (*Cynodon* spp.), buffalograss (*Buchloe* spp [Nutt.] Engelm.), and zoysiagrass (*Zoysia* spp. Willd.). Bermudagrass is a highly heterozygous and largely self-incompatible outcrossing plant (Burton and Hart 1967; Tan et al. 2014) with an estimated average 1X genome size of ~540 Mbp (Bethel et al. 2006; Taliaferro et al. 1997). *Cynodon* spp., including common bermudagrass, exhibit a series of ploidy levels (de Wet and Harlan 1970; Gulsen et al. 2009; Kang et al. 2008; Wu et al. 2006) with a basal chromosome number of 9 (Forbes and Burton 1963; Harlan and et al. 1970). Warm-season turfgrasses lag other crop species in the use of molecular and genomic approaches (Ebina et al. 2014; Hanna et al. 2013). This disconnect is particularly evident in Chloridoids, a clade with

~1,300 species, in which very few genetic linkage maps and QTL analyses have been reported (Bushman and Warnke 2013; Cai et al. 2014; Crawford et al. 2016; Ebina et al. 2014; Guo et al. 2016). Most efforts to date have been invested in zoysiagrass (Cai et al. 2014; Guo et al. 2014), and tef [*Eragrostis tef* (Zucc.) Trotter] (Hewan Demissie and Tatsuhito 2010; Yu et al. 2007; Zeid et al. 2010), and includes a whole genome draft sequence of tef (Cannarozzi et al. 2014) and zoysiagrass (Tanaka et al. 2016). Currently, *Cynodon* spp. lags these other chloridoids in genomic data and molecular breeding applications.

Bermudagrass Uses and Breeding Objectives

The *Cynodon* genus carries a suite of important characteristics that make it suitable for multifaceted end-uses with huge economic and ecological significance. Its resilience and fast recuperative potential, perenniality and aggressive sod-forming growth habit, and high resource use efficiency makes it an excellent multipurpose species that is widely used as a lawn and sports turf (e.g., on football fields and golf courses), livestock feed (i.e., for hay, forage, and pasture), and ground cover (i.e., to stabilize ditchbanks, roads, levees, and marginal lands). It exhibits a wide range of variation for plant characteristics, excellent adaptation and increased biomass in the warmer climates, traffic (wear) tolerance, aggressive growth and recuperative capacity, low input requirements, and drought and salinity tolerance (Beard 1972; Beard and Green 1994; Carrow and Duncan 1998; Hanna et al. 2013; Marcum 2008; Taliaferro 2003). Several triploid hybrids from interspecific crosses between *C. dactylon* (2n = 4x = 36) and *C. transvaalensis* Burtt Davy (2n = 2x = 18; South African bermudagrass) have found tremendous commercial success as lawn or sports turf (e.g., football fields and golf courses) (Florkowski and He 2008; Hanna and Anderson 2008), primarily because they combine stress tolerance of the tetraploid with aesthetic properties of the diploid (Hanna et al. 2013; Taliaferro 2003). Ironically, these same characteristics render it invasive, highly competitive, and an expensive weed to manage. Recently, bermudagrass has attracted research interest as a biomass feedstock for biofuel production (Xu et al. 2011), as a phytoremediation agent for soil reclamation from heavy metals (Elekes et al. 2010), petroleum (Razmjoo and Adavi 2012) and salinity (Cerda et al. 2007); and as a medicinal plant for its pharmacognostic properties (Rai et al. 2010). Current varietal development is largely

focused on tetraploid cytotypes (2n = 4x = 36) for forage and pasture, while both tetraploid and triploid cytotypes (2n = 3x = 27) are used in turf breeding (Wu 2011).

Bermudagrass morphological traits are routinely used to assess phenotypic diversity (Anderson 2005; Si-Yong et al. 2008; Wu et al. 2007), to develop core collections (Anderson 2005; Anderson et al. 2009; Jewell et al. 2012), and to characterize novel germplasm or cultivars (Lu et al. 2009; Taliaferro 2003). Owing to their high heritabilities (Wofford and Baltensperger 1985), morphological traits can be used as selection indices in breeding programs as proxies for correlated, yet much more complex traits (i.e., biomass, abiotic stress tolerance). For example, canopy height, an evaluation of shoot vertical growth, constitutes a significant component of vegetative performance (i.e., biomass, growth rate and habit) (Pittman et al. 2015; Sripathi et al. 2013; Wu et al. 2007) and can find use as an indirect selection index for improved pasture, biomass, and turf characteristics. Similarly, selection of improved wear (or traffic) tolerance can be achieved indirectly by selecting for morphological traits such as internode length (Wood and Law 1974), leaf length (Kowalewski et al. 2015), and leaf width (Kowalewski et al. 2015; Shearman and Beard 1975). Preliminary findings of Kowalewski et al. (2015) suggest that shorter and finer leaves result in increased wear tolerance in bermudagrass. Similarly, stolon growth rate and stolon length were found to be good predictors of establishment speed in *Cynodon* spp. (Magni et al. 2014), *Zoysia* spp. (Patton et al. 2007), and creeping bentgrass (Jones and Christians 2012).

Selection of improved bermudagrass cultivars is based on a number of morpho-phenological traits, growth attributes, and agronomic characteristics (including biotic and abiotic adaptations), and depends upon the intended end-use of the cultivar (for example, as pasture or turf) (Taliaferro 2003). While breeding bermudagrass for forage has primarily relied on traditional practices (i.e., mass selection, recurrent selection), mostly to develop seeded varieties of *C. dactylon* var. *dactylon* (Taliaferro 2003), successful turfgrass breeding programs have utilized both seeded and vegetatively propagated interspecific crosses (between *C. dactylon* and *C. transvaalensis*) as well as natural and induced mutations (Hanna et al. 2013; Wu 2011). Rapid, vertical shoot growth is desirable in pasture for rapid regeneration and increased biomass

production, while slow-growing, dwarf or prostrate, drought-resistant varieties are preferred in turf bermudagrass (Mutlu et al. 2015) to reduce mowing and irrigation requirements (Chen et al. 2009; Lu et al. 2008). A number of turf-bermudagrass genotypes, particularly used in golf greens, are spontaneous and irradiated dwarf mutants, particularly those of *C. dactylon x transvaalensis* interspecific hybrids. However, genetic and molecular architecture of dwarf mutation(s) in bermudagrass has not been elucidated (Abernathy 2014). Morphologically, short-statured plants, particularly dwarf mutants of bermudagrass and other grasses, have characteristically shorter leaves and internodes compared to their taller counterparts (Burton et al. 1969; Chen et al. 2016; Lu et al. 2009; Wu et al. 2007). Biologically, plant height is a sum of internode lengths from the base to the uppermost internode plus the length of the spike. Canopy height, on the other hand, encompasses stature-related features including vertical leaf growth, and is therefore a foliage characteristic. Accordingly, significant correlations have been reported among plant height/canopy height, internode length, and leaf length in bermudagrass (Wu et al. 2007) and allied lineages (Cui et al. 2011; Cui et al. 2002; Yu et al. 2014, Sripathi et al. 2013; Zeid et al. 2011). These traits often display continuous phenotypic variation, typical of multigenic control, and exhibit quantitative inheritance, which poses a substantial challenge in bermudagrass breeding. Nevertheless, significant genetic components and moderate to high heritability of these traits permit improvement of both turf and forage bermudagrass by breeding (Wofford and Baltensperger 1985; Wu et al. 2007).

Bermudagrass Genomic Resources

A paucity of DNA marker systems has hindered molecular genetics applications in bermudagrass - there is a need for a critical volume of reproducible and highly polymorphic markers like simple sequence repeats (SSR). Few studies have developed and reported bermudagrass genomic resources suitable for mining and development of SSR markers (Kim et al. 2008; Kamps et al. 2011; Tan et al. 2014). In fact, bermudagrass sequences in the public domain have already been mined for SSR development, genetic diversity, and pedigree analysis (Harris-Shultz et al. 2010a; Jewell et al. 2010; Wang et al. 2010; Harris-Shultz et al. 2011; Kamps et al. 2011; Tan et al. 2012; Zhiyong et al. 2013; Tan et al. 2014; Guo et al. 2015),

and also for the addition of markers to linkage maps (Bethel et al. 2006; Harris-Shultz et al. 2010a; Harris-Shultz et al. 2010b). While cross-taxon application of heterologous SSR in bermudagrass has been limited (Wang et al. 2005; Tan et al. 2012), homologous expressed sequence tags (ESTs) from bermudagrass yielded a lower frequency of SSR markers than ESTs from other grass species (Kantety et al. 2002; Varshney et al. 2002; Kim et al. 2008; Tan et al. 2012). For example, *in silico* mining of EST databases of major cereal species showed that around 3% of non-redundant ESTs harbored effective SSR for each database (Kantety et al. 2002; Varshney et al. 2002), while only up to 1.5% of unique ESTs were reported to contain effective SSR in bermudagrass databases (Kim et al. 2008; Tan et al. 2012). Nevertheless, preexisting heterologous PPs provide cost-efficient alternatives to *de novo* marker development, particularly when cross-transferability between the species is high. Further, heterologous markers leverage comparative genomic studies. For example, heterologous cDNA from *Pennisetum* and rice mapped in bermudagrass (Bethel et al. 2006) and sorghum (Bowers et al. 2003) aided in comparative mapping between the two species.

While bermudagrass molecular marker research has shed some light on genetic diversity (Harris-Shultz et al. 2011; Kamps et al. 2011; Kang et al. 2008; Ling et al. 2012; Wu 2011; Wu et al. 2004; Wu et al. 2006; Zhiyong et al. 2013), information on structural genomics is critically low. Little is known about bermudagrass genome constitution and evolution (i.e., origin, nature of polyploidy, duplication and divergence), and chromosome behavior during meiosis (i.e., inheritance), each of which are important for evolutionary studies, genetic linkage analysis, quantitative trait loci (QTL) detection, and crop improvement. To date only three papers have reported the construction of genetic linkage maps in bermudagrass, two of which report addition of small numbers of EST-derived markers to the only available framework. The first genetic linkage framework of bermudagrass (*C. dactylon x transvaalensis*) (Bethel et al. 2006) was based on single-dose restriction fragments (SDRFs) (Wu et al. 1992) generated from probes based on bermudagrass genomic clones and heterologous cDNAs from *Pennisetum* and rice. Triploid (2n = 3x = 27) F_1 progeny were used for mapping, based on heterozygosity within each parent, following a

pseudo-testcross mapping strategy (Grattapaglia and Sederoff 1994). The T89 map was based on 155 SDRFs and 17 double-dose restriction fragments (DDRFs), while that of T574 was based on 77 SDRFs. Harris et al. (2010b) added twenty-one bermudagrass-resistance gene analogs (BRGA) and EST-SSRs to the published (Bethel et al. 2006) SDRF framework. The group further updated the map with an addition of 28 EST-SSRs mined from cDNA pseudo-molecules developed at Plant Genome Mapping Laboratory (Kim et al. 2008). Further, based on disomic segregation of fourteen EST-SSRs and almost disomic profile at yet another marker locus (single inconsistent progeny), Harris-Shultz et al. (2010a) suggested that basic chromosomes pair preferentially during meiosis and that tetraploid bermudagrass (i.e., T89) could be a segmental polyploid. Recently, Guo et al. (2015) proposed allopolyploidy of common bermudagrass based on marker segregation analysis and Gong et al. (2013) made the same proposal based on fluorescence *in situ* hybridization (FISH) and meiotic chromosome configurations.

The genome of another chloridoid, tef [*Eragrostis tef* (Zucc.) Trotter], has been sequenced (Cannarozzi et al. 2014), but due to lack of a complete and saturated linkage map, comparative genomic assessment was inadequate to delineate chromosomal rearrangements during its evolution. However, high-density sequence-tagged genetic maps of zoysiagrass (Huang et al. 2016; Wang et al. 2015a) and finger millet (Srinivasachari et al. 2007) have yielded novel insights into chloridoid genome evolution. Densely-populated sequence-tagged saturated linkage maps are needed to improve knowledge of genome structural diversity in *Poaceae* that lack genome sequences. Accordingly, a genetic linkage map in prairie cordgrass (*Spartina pectinate* Link), another chloridoid with bioenergy potential, was constructed and compared with sorghum based on homologous tef genome scaffolds (Crawford et al. 2016). Further, high-resolution genetic maps can improve genome assembly by anchoring *de novo* sequences and providing a framework to orient and order smaller scaffolds into chromosome-scale pseudomolecules (Bartholomé et al. 2015b; Fierst 2015). For example, the validity of second chloridoid genome to be sequenced and assembled (i.e., *Zoysia japonica* accession 'Nagirizaki') was authenticated and pseudo-chromosomes of the species were

constructed (Tanaka et al. 2016) based on genetic linkage map from Wang et al. (2015a). However, no finished chloridoid genomes are reported; nevertheless, draft genomes are publicly available.

The development of linkage maps for many outcrossing species, including the framework linkage map of bermudagrass, relied on the "pseudo-testcross" strategy (Grattapaglia and Sederoff 1994) that involves separate estimation of parental linkage phases and recombination fractions, often using MapMaker/Exp (Lander et al. 1987). Wu et al. (2002a) developed maximum-likelihood methods for simultaneous estimation of linkage and linkage phases, which have been successfully applied to several outcrossing species including sugarcane (Garcia et al. 2006; Oliveira et al. 2007; Palhares et al. 2012), passion fruit (Oliveira et al. 2008; Pereira et al. 2013), rubber tree (Souza et al. 2011), eucalyptus (Bartholomé et al. 2015a), and switchgrass (Lowry et al. 2015). In the current study, application of a maximum-likelihood approach was extended to bermudagrass using functions implemented in the R software package OneMap (Margarido et al. 2007).

Objectives: Bermudagrass Research

Expressed sequence tags from cDNA show higher levels of sequence conservation across closely related species than random genomic DNA, making them amenable to cross-taxon applications (Ellis and Burke 2007). A number of studies have successfully used sugarcane EST-SSR for phylogenetic and molecular diversity studies among different grass species (Cordeiro et al. 2001; Lu et al. 2012; Sharma et al. 2008). Our laboratory has reported on application of sugarcane EST-derived SSR markers in cross-taxon mapping studies in *Miscanthus* spp. Aderss. (Kim et al. 2012) and *Sorghum* spp. Moench. (Kong et al. 2013). In the first part of our bermudagrass research, we report on rapid and robust cross-taxon application of sugarcane EST-SSR to characterize historically important 'Tif' series cultivars and bermudagrass accessions from the long-standing turfgrass breeding program at the Coastal Plain Experimental Station, Tifton, Georgia.

In the second part of our bermudagrass research, we report the construction of complete SSR-enriched linkage maps of two bermudagrass species, *C. dactylon* x *C. transvaalensis* using the same full-

sib progeny used to construct the RFLP-based framework (Bethel et al. 2006) and present an inferred ancestral chromosome complement based on alignment of the parental maps. Further, we leverage molecular approaches to assess the nature of ploidy in the tetraploid parent (i.e., T89). Additionally, we report comparative analysis with grass genome models (i.e, sorghum and rice) and four chloridoid species [i.e., tef (Cannarozzi et al. 2014), prairie cordgrass (Crawford et al. 2016), finger millet (Srinivasachari et al. 2007), and zoysiagrass (Wang et al. 2015a)] and deduce rearrangements that differentiate the modern *Cynodon* chromosomes from those of the polyploid common ancestor of Poaceae.

The updated bermudagrass map reported in our second bermudagrass study was suitable for exploratory QTL analysis since most of the parental genomes (i.e., ~ 80%) lie within 10 cM of a mapped marker (Khanal et al. 2017b), allowing adequate power for QTL detection (Lander and Botstein 1989). We also showed that tetraploid bermudagrass 'is in a relatively advanced state of diploidization' and there appears to have 'substantial subgenome differentiation', 'strong preferential pairing', and 'disomic inheritance', essentially providing a basis to use QTL mapping approaches used in the diploids. In the third part of bermudagrass research, we detect and characterize bermudagrass QTL for one foliar (i.e., canopy height), two stolon (i.e., stolon internode length and length of the longest stolon), and two leaf traits (i.e., leaf length and leaf width).

Cotton in a Nutshell

Cultivated cotton, a high value commodity crop of the arid and semi-arid tropics, is world's leading source of natural fiber and one of the most important sources of oil seeds. It belongs to the family of Mallows and genus *Gossypium* L., which represents 45 diploid and 5 allopolyploid extant species (Fryxell 1992; Percival et al. 1999; Vollesen 1987). Diploid *Gossypium* spp. have 13 pairs of chromosomes (2n=26), divided into eight different genome types designated as A, B, C, D, E, F, G, or K, and distributed in African, Asian, Australian, and North American tropics and subtropics (Endrizzi et al. 1985; Percival et al. 1999; Wendel et al. 1999). On the other hand, the allopolyploids evolved in the New World and have 26 pairs of chromosomes (2n=52) with complete A and D chromosome complements in their somatic cells (Wendel

and Albert 1992). Two old world diploids (*G. arboreum* and *G. herbaceum*) and two new world allopolyploids (*G. hirsutum* L. and *G. barbadense* L.) are domesticated and cultivated for their spinnable fibers. However, Upland cotton (*G. hirsutum*) alone accounts for more than 90% of global cotton production followed by Pima (also Egyptian; *G. barbadense*) cotton which contributes around 9% of the total (Abdurakhmonov et al. 2012). In the United States, cotton is planted in around 12 million acres, which makes it the fourth biggest crop industry contributing around $6 billion annually to the US economy (www.cotton.org).

Upland cotton accessions in the United States are classified into four distinct breeding pools, namely *Acala*, *Plains*, *Delta*, and *Eastern*, representing cultivars adapted to four partially overlapping production regions of the country (Niles and Feaster 1984). *Acala* types have superior fiber and spinning qualities and are better adapted to the irrigated Southwest. The second breeding pool constitutes *Plains* types which roughly cover one half of the US cotton belt and are mostly suited to Texas, Oklahoma, and eastern New Mexico. The third, *Delta* types are primarily grown in the rain-belt area from southern Texas to Alabama and cover approximately one third of the US cotton production area while *Eastern* types are mostly adapted to Southeastern states of Georgia and the Carolinas.

Fiber Properties

Although higher yields and yield stability remain top priorities for cotton improvement in the US, the paradigm has shifted to include better fiber quality in responses to the evolution of efficient spinning technologies and to the formidable challenge posed by the synthetic fibers, which created export directed demands for high quality cotton fibers. Since the quality of raw fiber determines yarn processing efficiency as well as the quality of the final produce (for example, textile), market-value of raw cotton is adjusted based on its quality. However, fiber quality is a collective term encompassing a suite of physical properties that are measured on fiber samples from a cotton bale (Bradow and Davidonis 2000). As such, several determinants dictate the market value of a bale of cotton. The major quality determinants used by textile industries for cotton pricing and quality control include length, strength, elongation, fineness, and

uniformity of the cotton fibers, which are measured by high-volume instrument (HVI) system (www.cottoninc.com). Some of the most important fiber properties pertinent to the proposed research are discussed:

Cotton lint fiber is the longest unicellular extension of a plant cell (Kim and Triplett 2001), the property which makes it a key attribute to the textile industry. HVI reports 'the upper-half-mean length' (UHML), which is basically the mean length of the longest 50% of fibers in a sample measured, in hundredths of an inch (www.cottoninc.com). UHMLs less than 0.99" is short, around 1.00" is medium, between 1.11" and 1.26" is long, and above 1.26" is extra long (www.cotton.org). Longer fibers produce better and stronger yarns as they have greater resistance to friction during the processing (Broughton et al. 1992).

Fiber strength is generally reported in grams per tex (grams/tex), which is the force required to break a bundle of sample fibers clamped 1/8 inch between the two sets of jaws. Grams/tex implies force (in grams) required to break a tex (weight in grams of 1,000 meters of fiber) of sample (www.cottoninc.com). Strength of less than 26 grams/tex is weak, between 26-29 grams/tex is medium, 30-32 grams/tex is strong, and above 33 grams/tex is very strong. Stronger fibers resist snapping during processing, which correlate with higher yarn strength (Chee and Campbell 2009).

Fiber elongation is a measure of elasticity in percentage, which is simultaneously measured when force is applied to break a bundle of sample fibers to find the fiber strength (Bradow and Davidonis 2000). In general, less than 5.9% elongation is low, 5.9% to 6.7% elongation is medium, 6.8% - 7.6% is high, and above 7.6% is very high. Higher elongation percent means higher yarn stretchiness which helps withstand fiber and yarn processing stresses, but does not directly contribute to yarn strength (May 2000).

Fiber fineness is indirectly measured as micronaire, which is an airflow measurement on a weighed fiber sample compressed to a specific volume in a chamber (www.cottoninc.com). Compressed fiber sample resist airflow and this resistance is proportional to the linear density of the fibers, which is expressed

in micrograms per inch and adjusted for the maturity of the fiber. Fiber fineness based market value adjustment considers a range of 3.7 to 4.2 mic as premium quality, ranges of 3.5 to 3.6 and 4.3 to 4.9 as base quality, and range of 3.7 to 4.2 as premium quality (www.cotton.org). Thick fibers result in coarse textile with lower thread counts, while very fine (immature) fibers adversely affect the spinning efficiency and fabric properties (Basra and Malik 1984; Grover and Hamby 1960; Hake et al. 1996). In fact, fiber fineness affects yarn strength more than the fiber strength (Sattar and Hussain 1985).

Cotton Gene Pool

Modern-day cotton varieties have a narrow genetic base (Tyagi et al. 2014; Wendel et al. 1992), which resulted from a series of genetic bottlenecks spanning a course of 1-2 million years since its evolution (Cronn et al. 2002). Transoceanic dispersal of an A-genome-like diploid from Africa (which is similar to modern *G. arboreum*) followed by its interspecific hybridization with a native D-genome-like diploid (which is similar to modern *G. raimondii*) subsequently to produce an AD-genome tetraploid (with A-genome cytoplasm) is perhaps the first major genetic bottleneck wherein variations within A- and D-genome species were severely curtailed in the incipient tetraploid (Wendel et al. 1989). Further, reproductive isolation after radiation of AD-genome tetraploids reduced intraspecific genetic variation and was exacerbated by domestication and selection by the early farmers. Consequently, a handful of landraces belonging to two species of AD-genome were selectively propagated, distributed, and retained over hundreds of years of cotton farming that formed the basis of modern-day varieties (Hutchinson 1947). However, scientific cotton breeding largely focused on developing high yielding varieties with broad environmental adaptability, which was accomplished through selection and reselection of lines derived from a few related genetic backgrounds (Bowman 2000). As a result, variation within cultivated cotton has severely depleted, while much of its extant gene-pool still remains unexplored.

Classically, the primary gene-pool of cultivated AD-genome cotton consisted of all cross-compatible AD-genomes including *G. tomentosum* Nuttall *ex* Seemann, *G. darwinii* Watt, and *G. mustelinum* Miers *ex* Watt as well as the wild, landrace, cultigens, and feral ecotypes of *G. barbadense* and

G. hirsutum (Khadi et al. 2010). Although AD-genome species are cross-compatible, natural hybrids or introgressed mosaics among sympatric species do not appear to persist and colonize over time and space, thereby limiting natural shuffling of favorable alleles among these genomes (Lehman et al. 2014; Pereira et al. 2012; Wendel et al. 1992). The reasons may be multilocus epistatic interactions and divergent gene regulatory systems restricting chromatin transmission (Jiang et al. 2000) or gene order rearrangements and cryptic chromosomal aberrations. As such, the four tetraploid species viz. *G. barbadense*, *G. darwinii*, *G. mustelinum*, and *G. tomentosum* could be reclassified as secondary gene pool for *G. hirsutum* (Lubbers and Chee 2009).

Introducing Novel Alleles from Secondary Sources

Cotton fiber properties show moderate to high heritability (May 1999; Meredith and Bridge 1972; Paterson et al. 2003), which supports early generation selection practiced by the cotton breeders (Bowman 2000). However, genetic gain for yield and fiber properties has reached a plateau in recent decades (Meredith 2000), which is mostly because of narrow breeding pools and exhaustion of sources of variation in breeding programs. Further, detection and fine mapping of the QTLs in intraspecific Upland mapping populations would require a critical mass of sub-genome specific SNPs, the generation of which would require high throughput resequencing and validation particularly because of lower levels of intraspecific molecular polymorphisms (Zhu et al. 2014).

On the other hand, introduction of novel allelic variation in cultivated gene pool from unadapted materials would require several generations before favorable alleles could be uncovered and utilized in selective breeding (Zhang et al. 2014). Although breeders have routinely introduced and shuffled exotic alleles from *G. barbadense* and *G. hirsutum* into the modern varieties (Mei et al. 2004; Zhang et al. 2014), systematic introgressions from *G. tomentosum*, *G. darwinii*, and *G. mustelinum* are only being explored in recent years (Waghmare et al. 2005; Wang et al. 2012a; Zhang et al. 2011). Linkage mapping, chromatin transmission studies, and QTL analysis in interspecific populations between *G. hirsutum* with *G. tomentosum* and *G. mustelinum* represent vital resources for introducing novel allelic variation in the

cultivated gene pool. In fact, although seemingly unfavorable donor parents for fiber quality alleles, both *G. tomentosum* and *G. mustelinum* contributed a number of favorable alleles for the traits. Also noteworthy is the preponderance of favorable alleles contributed by *G. tomentosum* for fiber fineness and elongation, and by *G. mustelinum* for fiber elongation - both traits with relatively short history of selection in scientific cotton breeding (Zhang et al. 2011). As such, introgression of favorable QTLs from *G. tomentosum* and *G. mustelinum* may offer avenues for rapid selection gains for these traits.

QTL Mapping for Fiber Quality Traits

The genetic dissection of complex traits into underlying QTLs provides opportunities to manipulate quantitative trait under Mendelian framework using marker-assisted selection (MAS) and QTL pyramiding strategies. Particularly in the case of cotton fiber quality, cost of phenotyping can be minimized while selection gain can be maximized by using QTL-MAS approaches. To date, hundreds of QTLs underlying fiber quality characteristics have been located, which apparently intersperse in the genetic linkage maps derived from crosses between different genetic backgrounds, tested in different environments, and are often tagged with different volumes and classes of genetic markers. Often, the size and nature of the mapping populations as well as the molecular markers hinder our ability to meta-analyze these maps and tailor the information to fine map, clone, utilize MAS, or pyramid favorable QTLs. Nevertheless, four meta-analysis have been published, which consolidated several genetic linkage maps into comprehensive maps with superimposed QTL data (Lacape et al. 2010; Rong et al. 2007; Said et al. 2013; Wang et al. 2013b). However, only a handful of public cotton breeding programs reportedly pursue QTL-MAS strategy to improve fiber quality traits (Hugie et al. 2014). In a recent review by Zhang et al. (2014), the authors could not find a single commercial cotton cultivar directly coming out of an interspecific introgression breeding between *G. hirsutum* and *G. barbadense*, which, ironically, is the most frequently pursued interspecific hybridization for Upland cotton improvement. In general, negative correlation between yield and quality and the stability of expression of fiber quality QTLs have remained the major concerns. Even significant QTLs (explaining high proportions of phenotypic variation) express precariously across different

environments, generations, and/or genetic backgrounds (Sun et al. 2012). QTLs that show stable expression to deliver significant genetic effects are amenable to molecular breeding. Accordingly, a number of fiber quality QTLs have been shown to have stable and significant genetic effects in different environments and backgrounds, suggesting the efficiency of MAS in breeding for enhanced fiber quality traits (Islam et al. 2014; Paterson et al. 2003; Sun et al. 2012; Wang et al. 2012b).

Colocalization of Fiber Quality QTL

An overarching result of the meta QTL analyses was the preferential distribution of fiber quality QTL in clusters and hotspots along specific genomic regions, which offer possibilities of multiple QTL introgression for simultaneous improvement of several fiber quality traits through MAS. A number of pleiotropic or close linkage relationships between favorable fiber quality QTL were also detected in *G. tomentosum* and *G. mustelinum*. Of interest, the location of a fiber elongation QTL (qFE14.1) corresponded with fiber fineness QTL (qFF14.1) from *G. tomentosum* and fiber length uniformity QTL (qFU14.1) from *G. hirsutum*, both of which showed significant genotype x background interactions and, interestingly, both were also detected in *G. hirsutum* x *G. barbadense* F_2 populations (Paterson et al. 2003). Similarly, a fiber elongation QTL (qFE21.1) co-located with fiber fineness QTL, qFF21.1. In *G. hirsutum* x *G. mustelinum* NIILs, fiber strength QTL (qSTR-25-1) co-located with fiber elongation QTL (qELO-25-1), which corresponds to evidences that common genetic loci may affect both traits (Chee et al. 2005a). Also, co-localization of QTL underlying different fiber quality traits may explain strong correlation among the phenotypes often reported in the literature (Zhang et al. 2011).

Epistasis, QTL Stacking, and G x E Interactions

A number of studies have underscored the importance of epistasis as a genetic basis of heterotic responses in cotton. Significant epistatic interactions have been reported for morphological and phenological traits (Guo et al. 2008; Liang et al. 2014; Liu et al. 2014; Song and Zhang 2009), yield and yield components (Jia et al. 2014), insect-pest and disease resistance (He et al. 2014; Ulloa et al. 2010), and

fiber quality traits (Saha et al. 2011; Yuan et al. 2014). Therefore, stacking/pyramiding fiber quality QTLs would be useful to test for epistatic interactions. Further, individual fiber quality QTLs often contribute relatively smaller proportions of the overall phenotypic variation (<10%) for the trait and are also influenced by the environment. As such, QTL pyramiding is desirable for most fiber quality traits.

To date, QTL pyramiding for fiber properties have exclusively focused on fiber length (Dong et al. 2009) and fiber strength (Guo et al. 2005; Yuan et al. 2014), and, in general, average lengths and strengths are reported to increase in the QTL stacked lines. Digenic interactions between two fiber strength QTLs were reported by Yuan et al. (2014). Further, fiber quality QTLs are significantly influenced by the backgrounds where these QTLs are deployed, suggesting the importance of multilocus interactions (Chee et al. 2005a; Chee et al. 2005b; Draye et al. 2005; Zhang et al. 2011).

Fiber quality traits also show significant environmental effects. Among different fiber properties, fiber elongation is most influenced by the environment (Wang et al. 2013a; Zhang et al. 2011). However, QTL studies report infrequent numbers of significant genotype x environmental interactions (Zhang 2011).

Objectives: Cotton Research

Backcross-selfed introgression lines carry interspecific alleles in a near-isogenic state. As such, "the phenotypic effect measured for each QTL is likely to be a better predictor of its ultimate effect when transferred to other cultivated backgrounds" (Chee et al. 2005b). Therefore, the objective of the proposed research is to assess the effects of a number of *G. tomentosum* and *G. mustelinum* QTLs transferred from near-isogenic introgression lines into cultivated *G. hirsutum* genetic backgrounds. Fiber elongation QTLs are specifically targeted together with fiber fineness, fiber strength, and fiber length QTLs for developing QTL-stacked segregating populations (details in methodology). These populations will help to decipher individual QTL effects and their interactions in different genetic backgrounds of Upland cotton.

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CHAPTER 2

CROSS-TAXON APPLICATION OF SUGARCANE EST-SSR TO GENETIC DIVERSITY ANALYSIS OF BERMUDAGRASS (CYNODON SPP.) [1]

[1] Khanal, S, Schwartz, B.M., Kim, C., Adhikari, J., Rainville, L.K., Auckland, S.A., and Paterson, A.H. 2017. *Genetic Resources and Crop Evolution* 64(8):2059-2070.

Abstract

Bermudagrass lags in genomic and molecular breeding resources, particularly regarding a critical mass of robust, reproducible, and highly polymorphic molecular markers like simple sequence repeats (SSR). Here, 2,017 sugarcane EST-SSR primer pairs (PPs) were screened for transferability and polymorphisms against commercial triploid hybrids and representatives of their parental species, tetraploid *Cynodon dactylon* and diploid *C. transvaalensis*. Fifty-four percent of PPs amplified target SSR in at least one of the two species, while 62% of these 'transferable' SSR were polymorphic. A subset of 228 polymorphic markers was utilized for genotyping 24 samples that included: 10 'Tif' series cultivars (i.e., released from Tifton, GA), their parental species, 10 F_1 hybrids between the two species, and two diverse *Sorghum* spp. A total of 90 and 144 PPs produced reproducible bands in the bermudagrass samples and the sorghum species, respectively. While 63 (70%) PPs were polymorphic among members of the 'Tif' diversity panel, 79 (54.8%) were polymorphic between the sorghum species. Further, 57 PPs polymorphic in the 'Tif' diversity panel were genotyped against 3 experimental lines and 7 commercial hybrids including 'TifTuf', a recent addition to the 'Tif' series. Joint analysis of 24 genotypes grouped bermudagrass accessions into two main clusters, one including *C. dactylon* and the other including *C. transvaalensis*. A small set of informative SSR readily differentiated the cultivar groups and identified potentially mischaracterized cultivars, but did not differentiate among mutants within cultivar groups. Heterologous EST-SSR from sugarcane are useful sources of polymorphic markers for cultivar identification in bermudagrass, and build on existing marker resources to facilitate genetic mapping, QTL analysis, and marker assisted selection.

Key words: *Cynodon*; Chloridoideae; SSR marker; transferability; polymorphism information content

Introduction

Bermudagrass is a common name referring to several resilient perennial grasses of the genus *Cynodon* (L.) Rich., which typically colonize warmer climates and are widely used for turf in the tropical, subtropical, and warm temperate regions of the world. The United States National Plant Germplasm System

(NPGS) records eight extant species and ten varieties, following the revised taxonomic classification by Harlan (1970a). The genus belongs to family Poaceae and subfamily Chloridoideae (Clayton and Renvoize 1986), in the "PACC" clade that contains subfamilies Panicoideae, Arundinoideae, Centothecoideae, and Chloridoideae (Kim et al 2009). While some Panicoids including maize, sorghum, and sugarcane have been extensively studied, Chloridoideae subfamily members lag in scientific exploration. Nonetheless, Chloridoideae is one of the most important subfamilies including a number of warm-season turfgrass species with the C4 photosynthetic pathway, such as bermudagrass (*Cynodon* spp.), buffalograss (*Buchloe* spp [Nutt.] Engelm.), and zoysiagrass (*Zoysia* spp. Willd.).

A paucity of DNA marker systems has hindered molecular genetics applications in bermudagrass - there is a need for a critical volume of reproducible and highly polymorphic markers like simple sequence repeats (SSR). Few studies have developed and reported bermudagrass genomic resources suitable for mining and development of SSR markers (Kim et al. 2008; Kamps et al. 2011; Tan et al. 2014). In fact, bermudagrass sequences in the public domain have already been mined for SSR development, genetic diversity, and pedigree analysis (Harris-Shultz et al. 2010a; Jewell et al. 2010; Wang et al. 2010; Harris-Shultz et al. 2011; Kamps et al. 2011; Tan et al. 2012; Zhiyong et al. 2013; Tan et al. 2014; Guo et al. 2015), and also for the addition of markers to linkage maps (Bethel et al. 2006; Harris-Shultz et al. 2010a; Harris-Shultz et al. 2010b). While cross-taxon application of heterologous SSR in bermudagrass has been limited (Wang et al. 2005; Tan et al. 2012), homologous expressed sequence tags (ESTs) from bermudagrass yielded a lower frequency of SSR markers than ESTs from other grass species (Kantety et al. 2002; Varshney et al. 2002; Kim et al. 2008; Tan et al. 2012). For example, *in silico* mining of EST databases of major cereal species showed that around 3% of non-redundant ESTs harbored effective SSR for each database (Kantety et al. 2002; Varshney et al. 2002), while only up to 1.5% of unique ESTs were reported to contain effective SSR in bermudagrass databases (Kim et al. 2008; Tan et al. 2012). Nevertheless, preexisting heterologous PPs provide cost-efficient alternatives to *de novo* marker development, particularly when cross-transferability between the species is high. Further, heterologous markers leverage

comparative genomic studies. For example, heterologous cDNA from *Pennisetum* and rice mapped in bermudagrass (Bethel et al. 2006) and sorghum (Bowers et al. 2003) aided in comparative mapping between the two species.

Expressed sequence tags from cDNA show higher levels of sequence conservation across closely related species than random genomic DNA, making them amenable to cross-taxon applications (Ellis and Burke 2007). A number of studies have successfully used sugarcane EST-SSR for phylogenetic and molecular diversity studies among different grass species (Cordeiro et al. 2001; Lu et al. 2012; Sharma et al. 2008). Our laboratory has reported on application of sugarcane EST-derived SSR markers in cross-taxon mapping studies in *Miscanthus* spp. Aderss. (Kim et al. 2012) and *Sorghum* spp. Moench. (Kong et al. 2013). Here, we report on rapid and robust cross-taxon application of sugarcane EST-SSR to characterize historically important 'Tif' series cultivars and bermudagrass accessions from the long-standing turfgrass breeding program at the Coastal Plain Experimental Station, Tifton, Georgia.

Materials and Methods

Plant materials

Most clonal bermudagrass varieties released as 'Tif' series from the cooperative agreement between the United States Department of Agriculture-Agriculture Research Service (USDA-ARS) and the University of Georgia Coastal Plain Experiment Station in Tifton are interspecific triploids between common bermudagrass (*Cynodon* dactylon (L.) Pers.) and African bermudagrass (*C. transvaalensis* Burtt-Davy). A total of nine historic 'Tif' series varieties, two common bermudagrass, and one African bermudagrass accession(s) were selected for assessing nucleotide diversity (hereafter referred to as the 'Tif' diversity panel) (Table 2.1). Two diverse parents of an interspecific *Sorghum* mapping population were also included to compare relative transferability and cross taxon utility of microsatellite markers between the Panicoid and Chloridoid subfamilies. Ten triploid progenies of a cross between a common bermudagrass ('T89') and an African bermudagrass ('T574') were included to ascertain reproducibility of the markers. A subset of polymorphic PPs were further screened against a set of 12 additional genotypes (hereafter referred

as ‘the second panel’), which included 7 commercial turf bermudagrass cultivars, 3 experimental lines, and two genotypes from ‘Tif’ diversity panel (i.e., ‘Tifgreen’, and ‘Tifway’) (Table 2.1).

‘Tif’ diversity panel (i.e., ‘Tiflawn’, ‘Tifway’, ‘Tifway II’, ‘Tifgreen’, ‘Tifgreen II’, ‘Tifdwarf’, ‘TifEagle’, ‘TifGrand’, ‘TifSport’, and ‘Tifton 10’) and the second panel (i.e., ‘Latitude 36’, ‘Northbridge’, ‘Patriot’, ‘Premier’, ‘Discovery’, ‘Celebration’, a ‘Tif” series variety ‘TifTuf’, and experimental lines ‘UGB-70’, ‘11-T-56’, and ‘09-T-31’) were obtained from B. Schwartz (University of Georgia, Tifton, GA). DNA of ‘T89’ (*C. dactylon*, 4x), ‘T574’ (*C. transvaalensis*, 2x), and ten F_1 T89 x T574 triploids were available from prior work (Bethel et al. 2006).

Genotyping

Bermudagrass sprigs were stored overnight at -80°C and lyophilized for 48 hr. DNA was extracted following the protocol of Aljanabi et al. (1999). Sugarcane EST-derived SSR used in this study were identified and reported in a prior study (Kim et al. 2012). A subset of 2,017 preexisting primer-pairs (Kim et al. 2012) was screened against T89 and T574 to assess the transferability of the markers (Supplemental File, Table S2.1). PCR was performed in 96- and 384-well plates. Reaction mixtures contained 1µL each of MgCl2 (3 mM), dNTPs (2 mM), and 10X PCR buffer (100 mM Tris-HCL at pH 9, 500 mM KCL, and 15 mM $MgCl_2$), 0.2µL of Taq DNA polymerase (1 U), 3.8µL of ddH_2O, and 2µL of template DNA (15 ng/µL) to constitute a total volume of 10µL PCR reaction. Typical cycling conditions for PCR were 95°C for 4 min in the first step, followed by 6 cycles of 94°C for 40 s, a gradient reaction from 58°C to 52°C each for 1 min, and 72°C for 1 min. The third step was 35 cycles of 94°C for 40 s, 55°C for 1 min, and 72°C for 1 min. After the last cycle, reactions were incubated at 72°C for final extension period of 10 min before cooling to 4°C. PCR amplicons were resolved by 10% non-denaturing polyacrylamide gel electrophoresis (PAGE), and visualized by staining with silver nitrate (Bassam et al. 1991). Transferable EST-SSR were scored for the presence or absence of bands in T89 and T574. A subset of transferable SSR that showed polymorphic profiles between the two genotypes was screened against the ‘Tif’ diversity panel. Further, PPs exhibiting polymorphism in the ‘Tif’ diversity panel were genotyped against the second panel.

Data analysis

Transferability was classified as clear or poor based on the clarity of the amplicons resolved in the PAGE gels and assessed relative to other markers and λ-DNA ladders. Reproducibility was assessed based on the amplification of two parental bermudagrass samples, 'T89' and 'T574', and ten F_1 individuals from a cross between them. Transferable and reproducible markers were used for diversity analysis in the 'Tif' diversity panel, excluding the mapping population and sorghum samples. Polymorphic markers were identified by the presence or absence of bands amplified by individual markers across the 'Tif' diversity panel. Band frequency, total number of monomorphic/polymorphic bands, and average number of bands per marker were recorded. The subset of PPs polymorphic in the 'Tif' diversity panel was also assessed in the second panel. 'Tifgreen', 'Tifway', and λ-DNA ladders (i.e., for size reference) common to both panels were used to ascertain allele correspondence between the two panels. Input files were prepared as binary data matrices wherein 1 represented presence and 0 represented absence of a particular band size or a null allele, respectively. The 'proportion of shared alleles' function of software tool PowerMarker V3.25 (Liu and Muse 2005) was used to calculate genetic distance matrices and to construct distance based dendrograms based on 100 bootstraps (Felsenstein 1985), which were resolved with software 'consensus' in the Phylip package (Felsenstein 1988) and visualized with TreeView (Page 1996). PowerMarker was also used to calculate major band frequencies (average frequencies of amplicons), gene diversity, and polymorphism information content (PIC). Principal coordinates analysis (PCoA) was performed using Microsoft Excel based software GenAlEx 6.41 (Peakall and Smouse 2006, 2012).

Results

Transferability and polymorphism

Out of a total of 2,017 PPs screened against *C. transvaalensis* (i.e., 'T574') and *C. dactylon* (i.e., 'T89'), 1,094 (54%) amplified at least one of the samples (i.e., transferable), 689 (34%) showed poor amplification, and 234 (12%) did not amplify (Supplementary File, Table S2.1). Of clearly transferable

markers, 1,071 amplified both and 23 appeared species specific (11 in 'T574' and 12 in 'T89'). Further, 676 (62%) were polymorphic, 264 (24%) were monomorphic, and 154 (14%) were ambiguous.

A subset of 228 clearly transferable and apparently polymorphic PPs were advanced to a second phase and genotyped against 'Tif' diversity panel (see "Materials and methods"). Among the twelve individuals (excluding the F_1s and *Sorghum* species) of the 'Tif' diversity panel, 90 PPs (41%) amplified 279 clear bands, of which 84 bands were monomorphic (Supplementary File, Table S2.2). In summary, 63 (70%) PPs amplified reproducibly polymorphic bands in the 'Tif' diversity panel.

Among the selected 228 PPs, 144 (63%) produced clear bands from at least one of the two *Sorghum* species. A total of 79 (34%) PPs amplified polymorphic bands, 65 (28%) produced monomorphic bands, 56 (24%) were poorly transferable, and 32 (14%) were not transferable to the *Sorghum* species. On average, transferable PPs amplified 1.9 bands; specifically, 69 (48%) amplified single bands, 44 (28%) amplified two bands, and 22 (15%) amplified three bands. Of 277 clear bands from the transferable markers, 142 bands amplified by 79 PPs were polymorphic between *S. bicolor* and *S. halepense*. A total of 67 (29%) PPs were clearly transferable to both *Sorghum* and *Cynodon* species.

Diversity among bermudagrass genotypes

In the 'Tif' diversity panel, 90 PPs amplified an average of 3 unique bands with a range of 1 to 9 reproducible amplicons - average frequency of the amplicons (average allele frequency) was 0.60±0.33. Most cross-transferable markers amplified either one (26.6%) or two (22.2%) bands in bermudagrass samples (Figure 2.1), while the polymorphic subset amplified an average of 4 bands per marker. Differences in band frequencies among different ploidy levels were not significant (data not shown). Between *C. dactylon* accession 'T89', and *C. transvaalensis* accession 'T574', a total of 221 unique bands were amplified with an average of 2.45 amplicons per marker. Averages of the gene diversity index (GD) and PIC values for all the PPs were 0.26 and 0.21, while those for the polymorphic subset were 0.30 and 0.24, respectively.

A genetic similarity matrix was derived from 'the proportion of shared bands' among the 'Tif' diversity panel amplified by 90 PPs (Supplemental File, Table S2.3). UPGMA cluster analysis formed two distinct groups characterized by the presence of common bermudagrass ('T89') in the first group and African bermudagrass in the second group (Figure 2.2a). 'Tifway' and its mutant derivative 'Tifway II' clustered with 'T89' while 'Tifgreen' and its mutant derivatives 'Tifdwarf' and 'TifEagle' clustered with 'T574'. 'Tifway', 'Tifway II', and 'TifSport' shared identical alleles at each locus amplified by 90 PPs, evident in the tree as a single cluster without any sub-clusters. Therefore, 'TifSport' appears to be a mutant of 'Tifway' or Tifway's mutant derivatives. 'Tifgreen', 'Tifdwarf', and 'TifEagle' also shared common alleles at all the microsatellite loci and formed a single undifferentiated cluster. Interestingly, 'Tifgreen II', reportedly a mutant of 'Tifgreen', formed a separate subcluster together with 'Tiflawn' within the 'T574' group. The novel shade tolerant hybrid, 'TifGrand' was closer to 'Tifway' than 'Tifgreen', where all three are interspecific hybrids from crosses between *C. dactylon* and *C. transvaalensis*. Two *C. dactylon* accessions, 'T89' and 'Tifton 10' did not cluster together; in fact, 'Tifton 10' (a hexaploid), clustered with the intraspecific *C. dactylon* hybrid, 'Tiflawn' (a tetraploid).

Of 63 polymorphic PPs identified in the 'Tif' diversity panel, 57 were used to screen the second panel (see "Materials and Methods"). First, gels were scored independent of the 'Tif' diversity panel and later, allele correspondence was ascertained based on common genotypes ('Tifgreen' and 'Tifway') and the size standard (λ-DNA ladder). In separate analysis of the second panel, the 57 PPs amplified an average of 4 unique bands with a range of 1 to 10 reproducible amplicons for a total of 227 amplicons – average frequency of the amplicons was 0.57±0.33 (Supplemental File, Table S2.4). Further, averages of the gene diversity indices (GD) and PIC values for the 57 PPs were 0.28 and 0.22, respectively.

A genetic similarity matrix was calculated for the second panel based on shared amplicons produced by 57 PPs (Supplemental File, Table S2.5). UPGMA formed three distinct clusters. One group carried the new 'Tif' cultivar 'TifTuf' clustering with 'Tifway' and an experimental line ('UGB-70') (Figure 2.2b). The second cluster carried *C. dactylon* cultivars 'Discovery' and 'Celebration' together with

another experimental line (‘11-T-56’). The third cluster grouped remaining interspecific hybrids including an experimental line (‘09-T-31’) with ‘Tifgreen’. A subcluster within the third group carried three turf bermudagrass cultivars released from Oklahoma State University Turf Grass Program (‘Patriot’, ‘Northbridge’, and ‘Latitude 36’) and cultivar ‘Premier’, a suspected ‘Tifgreen’ mutant.

Joint analysis of the diversity panels was based on a total of 183 bands (143 polymorphic and 40 monomorphic) amplified by 54 common PPs among 22 bermudagrass accessions (Supplemental File, Table S2.6). Averages of the gene diversity indices (GD) and PIC values for the 54 PPs were 0.29 and 0.23, respectively; hence, summary stats (for example, GD, PIC etc.) in separate analyses were comparable to the joint analysis. UPGMA clustering was based on a genetic similarity matrix derived from ‘the proportion of shared bands’ in the joint panel (Supplemental File, Table S2.7). Genotype relationships were conserved among UPGMA clustering of the separate (Figure 2.2a and 2.2b) and joint panels (Figure 2.2c). Principal coordinates analysis (PCoA) supported the UPGMA clustering (Figure 2.3). The two major clusters in the UPGMA tree were separated along the first principal coordinate explaining 28.9% of total variance. The cumulative variances including the second and third coordinates reached 41.5 and 50.7% of total variation, respectively.

Individual and group specific alleles

Genotyping data showed a number of individual and group specific amplicons that can be used for cultivar identification (Supplemental File, Tables S2.2, S2.4, S2.6). ‘Tifway’, ‘Tifway II’, and ‘TifSport’ formed an undifferentiated cluster that shared a greater proportion of alleles with ‘T89’ (0.92) than with ‘T574’ (0.75). ‘Tifgreen’, ‘Tifdwarf’, and ‘TifEagle’ formed another undifferentiated group that shared a greater proportion of alleles with ‘T574’ (0.93) than with ‘T89’ (0.83). Differential allelic composition was further evident from the distribution of presence-absence amplicons from the two diploid species to the two cultivar groups (Table 2.2). The ‘Tifgreen’ group shared 20 T574-specific alleles and only 8 T89-specific alleles, while the ‘Tifway’ group shared 39 T89-specific alleles and only 12 T574-specific alleles. Further, the ‘Tifway’ group and ‘TifTuf’ carried 1 unique allele (not from ‘T574’ or ‘T89’), while ‘TifGrand’ carried

2, suggesting that the parental accessions are true representative of the original parental lines that were crossed to develop these cultivars. The remaining interspecific hybrids carried 10 to 18 unique alleles, indicating that one or both parental accessions could be different from used in the present study.

Discussion

Transferability and polymorphism of novel EST-SSR in bermudagrass

Cross-taxon transferability of SSR is closely related to evolutionary or phylogenetic relationship among grasses (Wang et al. 2005). In general, members of Poaceae family show high transferability rates (more than 50%). For example, Saha et al. (2004) reported 57% transferability of tall fescue EST-SSR across a diverse set of forages and cereals, almost two-fold higher than transferability of sugarcane SSR to bermudagrass and sorghum. Nevertheless, around 50% of sugarcane markers were clearly transferable to common bermudagrass (*C. dactylon*) and/or African bermudagrass (*C. transvaalensis*). However, compared to similar cross-taxon studies involving heterologous SSR in *Cynodon* spp., the rates of transferability reported herein are lower. Wang et al. (2005) reported an average transfer rate of around 53% for EST-SSR from much more diverse grasses to *Cynodon dactylon*. Similarly, Tan et al. (2012) reported 61% of genomic SSR transferable from sorghum to the two species of bermudagrass; albeit, only 44% of bermudagrass-based EST-SSR produced reproducibly amplifiable bands in the bermudagrass samples. One of the reasons for lower transferability in this study is the use of clarity as an objective parameter in determining transferability. Thirty-four percent of markers scored as poorly transferable amplified faint bands, characteristic of base-pair mismatches at the primer binding sites. Only 12% of the markers did not amplify the bermudagrass samples at all, which may be due to contraction or expansion of regions intervening between the SSR primers in the bermudagrass genomes or to indel polymorphisms at the primer binding sites.

Primer pairs used in this study were a subset of sugarcane EST-SSR that showed 100% sequence conservation against the *Sorghum bicolor* reference genome, and were used to construct a *Miscanthus* map (Kim et al. 2012). Although the reference genotype of ‘BTx623’ (Paterson et al. 2009), one of the *Sorghum*

species included in this study, was used for determining sequence conservation, only 63% of PPs amplified clear bands in the *Sorghum* species. Almost twice the number of sugarcane markers was transferable to *Miscanthus* (Kim et al. 2012) than bermudagrass (current study), consistent with much closer relationship between *Miscanthus* and *Saccharum* species (Paterson et al. 2013). Similarly, Tan et al. (2012) reported more than two-fold differences in transferability of genomic SSR from sorghum to *C. transvaalensis* and two *C. dactylon* cytotypes, which was not evident in the current study, indicating comparatively greater sequence conservation at the genic (EST-based) versus the random genomic regions.

In this study, about 62% of transferable markers were polymorphic between two species of bermudagrass. Wang et al. (2005) reported a lower frequency (57%) of polymorphic sorghum-transferred EST-SSR markers among bermudagrass species. Such discrepancies are routinely associated with the choice of genotypes, as well as resolution systems for amplified PCR products which offer different separation sensitivities. Specifically, we used PAGE gels that offer greater sensitivity than the agarose gels used by Wang et al. (2005). Also, different levels of nucleotide polymorphism have been reported in *Cynodon* spp. depending on the specific germplasm studied (Jewell et al. 2010; Kamps et al. 2011; Ling et al. 2012; Zhiyong et al. 2013). For example, Kamps et al. (2011) screened a total of 25 PPs developed through a genomic microsatellite enrichment procedure in a comparable panel (*C. dactylon*, *C. transvaalensis*, and 12 bermudagrass cultivars) and reported more than threefold higher PIC for the genomic SSR (0.817) than that obtained in this study. In partial summary, these results are in line with the theoretical and empirical understanding that EST-SSR are more transferable, but less polymorphic, than their genomic counterparts.

Although more alleles were amplified in tetraploid ('T89') than diploid African bermudagrass ('T574'), band frequencies across different ploidy levels were not correlated, reiterating similar results from Tan et al. (2012). However, Gulsen et al. (2009) studied 182 accessions with different ploidy levels and found a weak positive correlation and a quadratic relationship among the ploidy levels and band

frequencies. The number and nature of samples in this study may not be adequate to represent the ploidy series in natural *Cynodon* populations.

Genetic diversity of turf bermudagrass

The utility of transferable EST-SSR to decipher nucleotide diversity among twenty-two bermudagrass accessions was assessed based on genetic distance estimates deduced from 'the proportion of shared bands' amplified by 54 to 90 PPs in separate and joint diversity analyses. Genetic similarity ranged from 0.51 to 0.98 with the lowest proportion of bands shared between the 'Tifgreen' group and common bermudagrass, 'T89', while the highest proportion was shared between two *C. dactylon* cultivars viz. 'Celebration' and 'Discovery'. In fact, 'Celebration' and 'Discovery' differed only at three marker loci amplified by three different PPs. Also, two interspecific cultivars, 'Northbridge' and 'Latitude', shared a large proportion of common alleles (0.93). 'Northbridge' (OKC 1134) and 'Latitude 36' (OKC 1119) are triploid hybrids with a common *C. transvaalensis* parent (OSU selection "2747") (Wu et al. 2013, 2014). The expected relatedness between 'Northbridge' and 'Latitude' is supported by DNA markers (Wang et al. 2010), including those in the present study. The current study also provides molecular evidence of a familial relationship between 'Patriot' and 'Tifton 10', which has been reported in prior studies (Wang et al. 2010; Wu et al. 2005).

Among the 'Tif' series, the highest proportion of alleles was shared between the 'Tifway' group and 'T89' (0.85) followed by the 'Tifway' group and a new drought-tolerant variety, 'TifTuf' (0.84). Further, 'Tifway' also shared a relatively high proportion of alleles with the shade tolerant cultivar, 'TifGrand' (0.80), which supports results from earlier studies (Chen et al. 2009; Harris-Shultz et al. 2010a; Wang et al. 2010). From our allele distribution study, we suspect that allelic variation in 'T574' and 'T89' is mostly inherited in the hybrid cultivars - ninety-four percent of alleles from the two parental representatives were also present in the hybrids. Nevertheless, most *C. dactylon* accessions included in the study ('T89', 'Tifton 10', 'Discovery', 'Celebration') as well as an intraspecific *C. dactylon* hybrid, 'Tiflawn', were dispersed in different clades during cluster analysis, suggesting wide genetic diversity

among accessions of this species. A similar observation was reported by Zhang et al. (1999). In nature, common bermudagrass inhabits an extensive geographic range, represents diploid, tetraploid (e.g., 'T89'), and hexaploid (e.g., 'Tifton 10') chromosome races (de Wet and Harlan 1970), and displays a wide spectrum of nucleotide variation (Wu et al. 2004, 2005). Accordingly, there appears to exist extensive additional variation within *Cynodon dactylon* that can be used to broaden the genetic base of turf bermudagrass.

Molecular studies have suggested errors in pedigree information for some of the most important bermudagrass cultivars. 'TifEagle' was originally reported as an irradiated mutant of 'Tifway II' (Hanna and Elsner 1999). However, a number of reports including this study grouped 'TifEagle' with 'Tifgreen' (Zhang et al. 1999; Harris-Shultz et al. 2010a; Wang et al. 2010). Similarly, 'TifSport' was originally reported as an irradiated mutant of 'Midiron' (Hanna et al. 1997). However, different marker systems including EST-SSR from this study have consistently grouped 'TifSport' with 'Tifway' (Chen et al. 2009; Harris-Shultz et al. 2010a). Also, a number of studies that included both 'TifSport' and 'Midiron' are unequivocal about 'Tifway' parentage of 'TifSport' (Zhang et al. 1999; Yerramsetty et al. 2008; Wang et al. 2010; Kamps et al. 2011). In this study, the genotype profile for 'Tifgreen II' at several microsatellite loci was distinct from its reported parent, 'Tifgreen', which may suggest yet another error in parentage information and needs further evaluation.

Genotype and group specific diagnostic alleles

Most microsatellite studies, with a few exceptions (Harris-Shultz et al. 2011; Kamps et al. 2011), were not able to discriminate among the mutants within the 'Tifgreen' and 'Tifway' cultivar groups (Harris-Shultz et al. 2010a; Wang et al. 2010; Kamps et al. 2011). Likewise, we could not find any polymorphisms within these cultivar groups. Nonetheless, we identified a number of SSR markers that could differentiate between the 'Tifgreen' and 'Tifway' groups, their parental species, and other cultivars included in this study. Off-types appear as conspicuous patches of morphological variants in bermudagrass greens, which may be due either to contamination or to chromosomal aberrations and genetic mutations (Caetano-Anollés

1998; Caetano-Anollés et al. 1997; Reasor et al. 2016). There is a large demand from the stakeholders in the bermudagrass industry to be able to distinguish spontaneous mutants from turf contaminants (Harris-Shultz et al. 2010a) and to maintain genetic purity of breeding lines and to establish pure stands of desired cultivars (Reasor et al. 2016). As such, a subset of markers developed in this study may help to identify true mutants and/or contaminants of golf greens. For example, 'Premier' was a clonal selection from a distinctly different vegetative patch identified in a 'Tifgreen' fairway (Parsons and Lehman 2007). The cultivar is characterized as a mutant derivative of 'Tifgreen' with an interspecific pedigree, further supported by the fact that it is reproductively sterile. In the current study, we found that 'Premier' differs from 'Tifgreen' at a number of SSR loci, but apparently inherits a large number of alleles both from *C. dactylon* and *C. transvaalensis*, which suggests that the cultivar is a chance hybrid between *C. dactylon* and *C. transvaalensis*, but does not appear to be a mutant 'Tifgreen'.

'Tifgreen' and 'Tifway' groups shared the majority of alleles with *C. dactylon* and *C. transvaalensis* included in this study, which represent the original progenitor species of these groups. 'Tifgreen' was initially selected from a heterogeneous triploid population derived from a cross between a common bermudagrass and an African bermudagrass species (Hein 1961), while 'Tifway' was probably a chance hybrid between the same two species (Burton 1960). Wu et al. (2005) suspected that bermudagrass hybrids might share larger number of alleles with the higher-ploidy parent. However, differential allelic distribution from the parental species to the 'Tifgreen' and 'Tifway' varietal groups was evident – the 'Tifway' group shared more alleles with common bermudagrass ('T89'), while the 'Tifgreen' group shared more alleles with African bermudagrass ('T574'). 'Tifway' parentage (i.e., parental accessions and direction of crosses) is clearly documented (Table 2.1) while that for 'Tifgreen' is missing. 'Tifway' shared more alleles with *C. dactylon*, its paternal species. 'TifGrand' and 'TifTuf' also shared more alleles with their paternal species (i.e., *C. dactylon*); other interspecific hybrids (i.e., 'Latitude 36', 'Northbridge', 'Premier') are inconclusive. Nevertheless, one or both accessions used in this study may not be the original parental species for any of the cultivars or cultivar groups included in this study, as is known to be the case

with ‘Latitude 36’, ‘Northbridge’, and ‘Patriot’. In that context, analysis of genetic diversity of a broader and deeper sampling of the *Cynodon* germplasm pool is warranted.

As the paucity of polymorphic markers in bermudagrass has hindered downstream applications such as rapid and robust varietal characterization, high-resolution linkage mapping, and QTL analysis, reproducible and polymorphic SSR reported in this study are a useful addition to the scant marker resources in bermudagrass. Accordingly, we are using these markers to build an integrated genetic map and for QTL analysis. Cross-taxon utility of these anchor markers will also allow us to compare Panicoid and Chloridoid genomes, providing better insights into the evolution of grass genomes.

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Table 2.1 List of genotypes included in the study.

Accession	Alias	Epithet	Ploidy	Reference
'Tif' diversity panel				
T574	PI 606545	*C. transvaalensis* Burtt-Davy	2n = 2x = 18	
Tifway	Tifway 419	*C. transvaalensis* x *C. dactylon* (L.) Pers.	2n = 3x = 27	Burton 1966a
Tifway II		*C. transvaalensis* x *C. dactylon*	2n = 3x = 27	Burton 1985
Tifgreen	Tifton 328	*C. dactylon* x *C. transvaalensis*†[a]	2n = 3x = 27	Hein 1961
Tifgreen II		*C. dactylon* x *C. transvaalensis*†	2n = 3x = 27	Burton 1991
Tifdwarf		*C. dactylon* x *C. transvaalensis*†	2n = 3x = 27	Burton 1966b
TifEagle	TW-72	*C. dactylon* x *C. transvaalensis*†	2n = 3x = 27	Hanna and Elsner 1999
TifGrand (PP21017)	ST-5 (Tift No. 4)	*C. transvaalensis* x *C. dactylon*	2n = 3x = 27	Hanna et al. 2010
TifSport	Tift 94 (PI 595365)	*C. transvaalensis* x *C. dactylon*	2n = 3x = 27	Hanna et al. 1997
T89		*C. dactylon*	2n = 4x = 36	
Tiflawn	Tifton 57	*C. dactylon* x *C. dactylon*	2n = 4x = 36	Hein 1953
Tifton 10	PI 539857	*C. dactylon*	2n = 6x = 54	Hanna et al. 1990
F_1-1 to F_1-10		*C. dactylon* x *C. transvaalensis*	2n = 3x = 27	Bethel et al. 2006
BTx623	PI 659985	*Sorghum bicolor* (L.) Moench.	2n = 2x = 20	NGPR, USDA 2013
Gypsum-9E		*S. halepense* (L.) Pers.	2n = 4x = 40	
Second Panel				
TifTuf	DT-1	*C. dactylon* x *C. transvaalensis*	2n = 3x = 27	Hanna and Schwartz 2016
Latitude 36		*C. dactylon* x *C. transvaalensis*	2n = 3x = 27	Wu et al. 2014
Northbridge		*C. dactylon* x *C. transvaalensis*	2n = 3x = 27	Wu et al. 2013
Patriot	OKC 18-4	*C. dactylon* (Tifton 10) x *C. transvaalensis*	2n = 4x = 36	Taliaferro et al. 2006
Premier		*C. dactylon* x *C. transvaalensis*	2n = 4x = 27‡[b]	Parsons and Lehman 2007
Discovery	BARAZUR; BAR 1CD3	*C. dactylon*	2n = 4x = 36	de Bruijn 2012
Celebration	Riley's Super Sport	*C. dactylon*	2n = 4x = 36	Riley 2000
UGB-70		*C. dactylon* x *C. transvaalensis*	2n = 3x = 27	Experimental line
11-T-56		*C. dactylon* x *C. transvaalensis*	2n = 3x = 27	Experimental line
09-T-31		*C. dactylon* (Tifton 10) x *C. transvaalensis*	2n = 4x = 36‡	Experimental line

[a] Direction of cross uncertain

[b] Ploidy level uncertain (not verified with cytological methods)

Table 2.2 Number of group specific amplicons shared among parental species (i.e., diploid *T574* and tetraploid *T89*), and the triploid hybrids.

Amplicon (source)	*C. transvaalensis* (T574)	*C. dactylon* (T89)	T89 and T574	Unique bands[a]
Tifgreen group[b]	20	8	91	18
Tifway group[c]	12	39	103	1
TifGrand	12	33	105	2
TifTuf	8	18	67	1
Latitude 36	11	11	54	10
Northbridge	13	8	55	10
Patriot	11	15	56	12
Premier	12	10	52	11
C. dactylon x transvaalensis hybrids	38	54	114	28
Unique bands[d]	8	4	1	-

[a] Bands not amplified in T574 or T89.
[b] Tifgreen, Tifdwarf, and TifEagle constitute Tifgreen group.
[c] Tifway, Tifway II, and Tifsport constitute Tifway group.
[d] Bands not amplified in the hybrids.

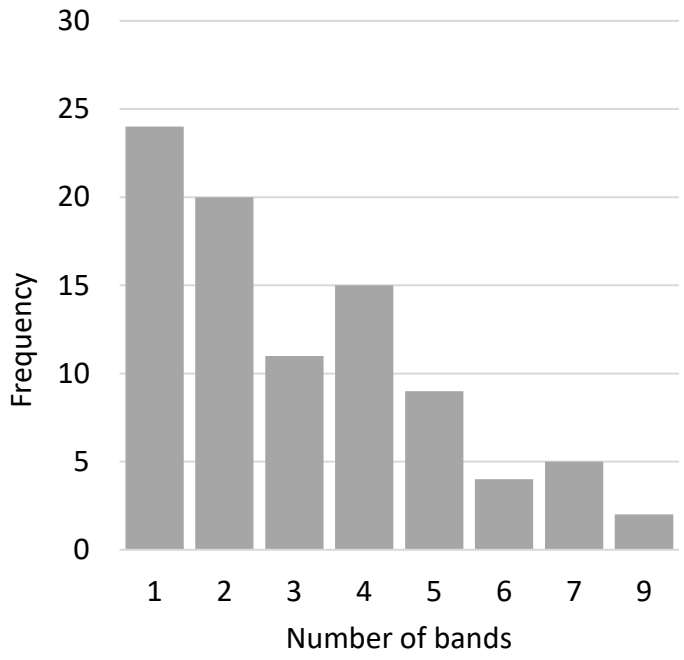

Figure 2.1 Distribution of number of bands amplified by 90 sugarcane EST-SSR primer pairs in 'Tif' bermudagrass panel with 12 accessions.

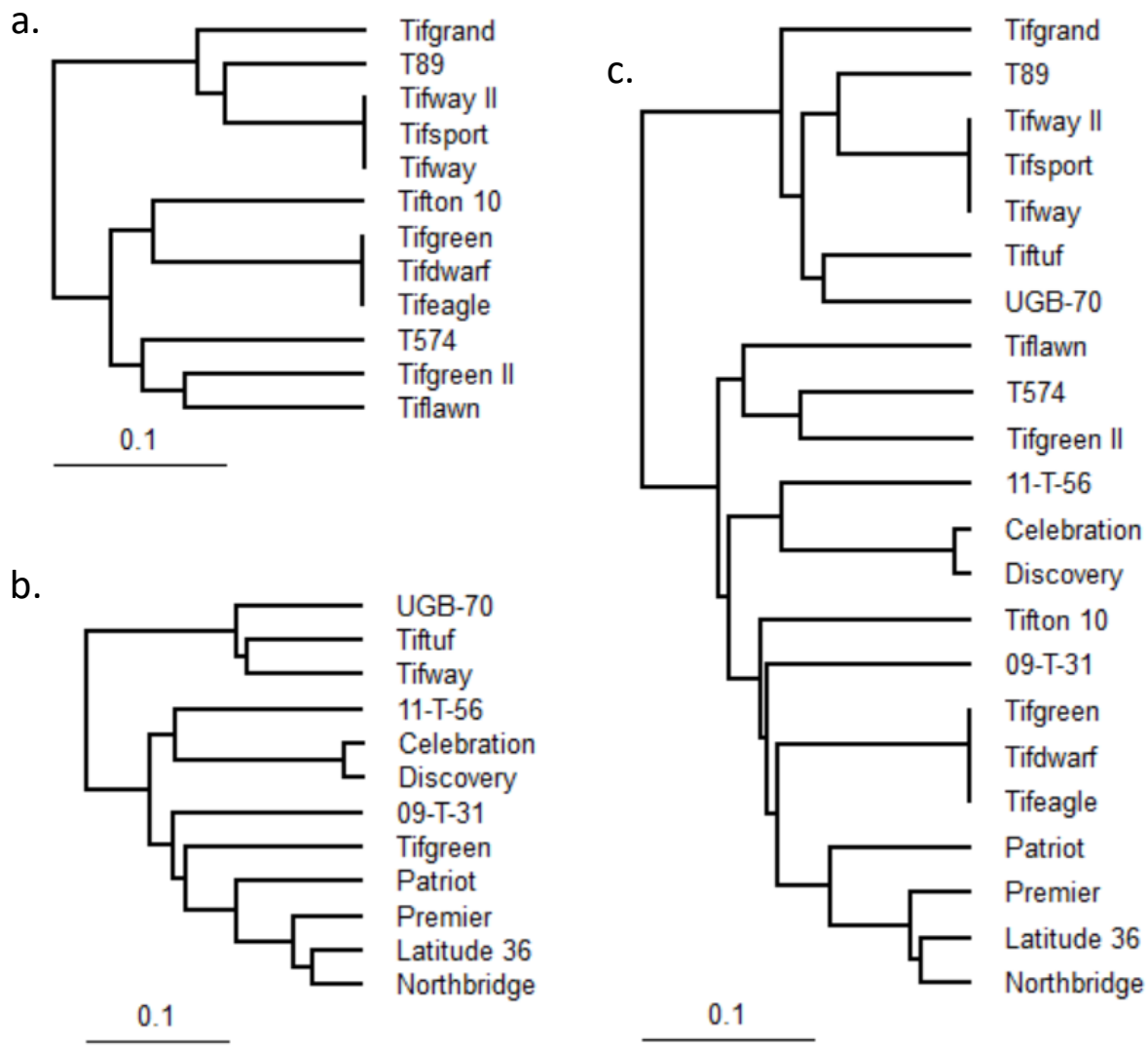

Figure 2.2 Dendrograms based on UPGMA cluster analyses of a. 279 bands amplified by 90 primer pairs across 'Tif' diversity panel (12 genotypes), b. 227 bands amplified by 57 primer pairs across second panel (12 genotypes), and c. Joint analysis based on 183 bands amplified by 54 markers across 22 genotypes.

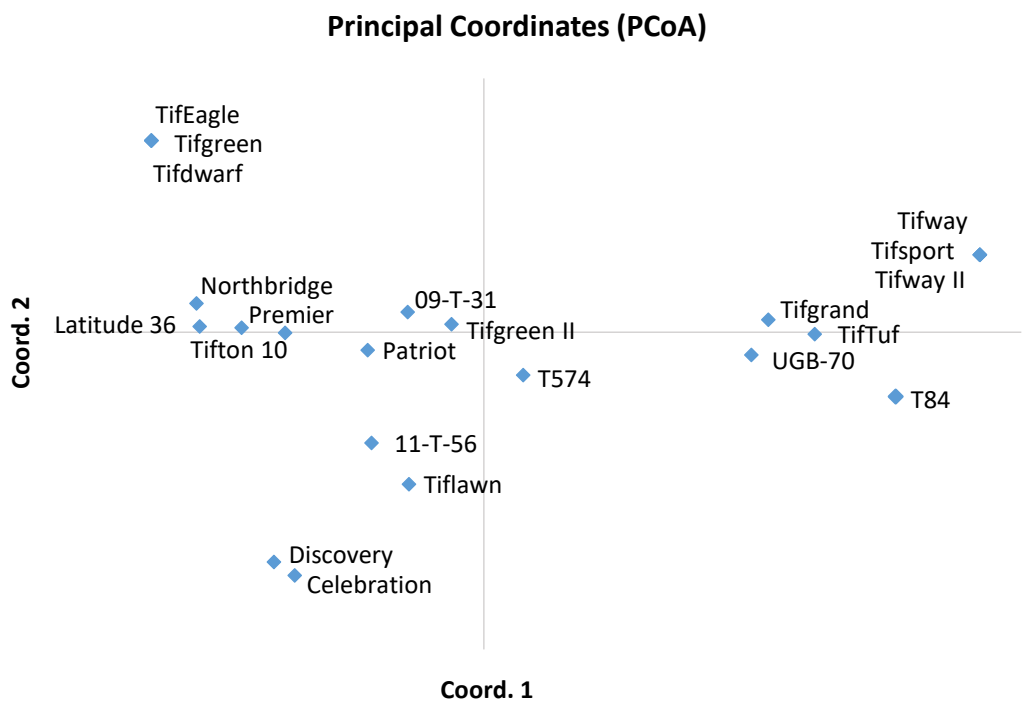

Figure 2.3 PCoA plot of the first two axes based on 183 bands amplified across 22 bermudagrass genotypes.

CHAPTER 3

SSR-ENRICHED GENETIC LINKAGE MAPS OF BERMUDAGRASS (*CYNODON DACTYLON* X *TRANSVAALENSIS*), AND THEIR COMPARISON WITH ALLIED PLANT GENOMES [2]

[2] Khanal, S, Kim, C., Auckland, S.A, Rainville, L.K., Adhikari, J., Schwartz, B.M., and Paterson, A.H. 2017. *Theoretical and Applied Genetics* 130(4):819-839.

Abstract

This study describes genetic linkage maps of two bermudagrass species, *Cynodon dactylon* (T89) and *Cynodon transvaalensis* (T574), that integrate heterologous microsatellite markers from sugarcane into frameworks built with single-dose restriction fragments (SDRFs). A maximum-likelihood approach was used to construct two separate parental maps from a population of 110 F_1 progeny of a cross between the two parents. The T89 map is based on 291 loci on 34 cosegregating groups (CGs), with an average marker spacing of 12.5 cM. The T574 map is based on 125 loci on 14 CGs, with an average marker spacing of 10.7 cM. Six T89 and one T574 CG(s) deviated from disomic inheritance. Further, marker segregation data and linkage phase analysis revealed partial residual polysomic inheritance in T89, suggesting that common bermudagrass is undergoing diploidization following whole genome duplication (WGD). Twenty-six T89 CGs were coalesced into 9 homo(eo)logous linkage groups (LGs), while 12 T574 CGs were assembled into 9 LGs, both putatively representing the basic chromosome complement (x = 9) of the species. Eight T89 and two T574 CGs remain unassigned. The marker composition of bermudagrass ancestral chromosomes was inferred by aligning T89 and T574 homologs, and used in comparisons to sorghum and rice genome sequences based on 108 and 91 significant blast hits, respectively. Two nested chromosome fusions (NCFs) shared by two other chloridoids (i.e., zoysiagrass and finger millet) and at least three independent translocation events were evident during chromosome number reduction from 14 in the polyploid common ancestor of Poaceae to 9 in *Cynodon*.

Keywords Chloridoideae, cosegregating group, EST-SSR, homo(eo)logous chromosomes, mode of inheritance, OneMap

Introduction

The genus *Cynodon* represents several perennial grasses that are widespread in tropical and subtropical landscapes, and commonly referred to as bermudagrass. Common bermudagrass [*Cynodon dactylon* (L.) Pers.] was introduced into the United States in the mid-1700's and within decades, it

naturalized in the warm regions of the country, becoming the most prominent pasture grass of the Southern states (Harlan 1970; Wu 2011). The *Cynodon* genus carries a suite of important characteristics that make it suitable for multifaceted end-uses with huge economic and ecological significance. Its resilience and fast recuperative potential, perenniality and aggressive sod-forming growth habit, and high resource use efficiency makes it an excellent multipurpose species that is widely used as a lawn and sports turf (e.g., on football fields and golf courses), livestock feed (i.e., for hay, forage, and pasture), and ground cover (i.e., to stabilize ditchbanks, roads, levees, and marginal lands). Ironically, these same characteristics render it invasive, highly competitive, and an expensive weed to manage. Recently, bermudagrass has attracted research interest as a biomass feedstock for biofuel production (Xu et al. 2011), as a phytoremediation agent for soil reclamation from heavy metals (Elekes et al. 2010), petroleum (Razmjoo and Adavi 2012) and salinity (Cerda et al. 2007); and as a medicinal plant for its pharmacognostic properties (Rai et al. 2010).

Bermudagrass is a highly heterozygous and largely self-incompatible outcrossing plant (Burton and Hart 1967; Tan et al. 2014) with C4 photosynthesis and an estimated average 1X genome size of ~540 Mbp (Bethel et al. 2006; Taliaferro et al. 1997). *Cynodon* spp., including common bermudagrass, exhibit a series of ploidy levels (de Wet and Harlan 1970; Gulsen et al. 2009; Kang et al. 2008; Wu et al. 2006) with a basal chromosome number of 9 (Forbes and Burton 1963; Harlan and et al. 1970). A number of leading turf varieties are sterile triploid hybrids from crosses between tetraploid common bermudagrass (*C. dactylon* L. Pers. var. *dactylon*) and a diploid African bermudagrass (*C. transvaalensis* Burtt Davy). Current varietal development is largely focused on tetraploid cytotypes (2n = 4x = 36) for forage and pasture, while both tetraploid and triploid cytotypes (2n = 3x = 27) are used in turf breeding (Wu 2011).

The genus *Cynodon* belongs to family *Poaceae* (*Gramineae*) and subfamily *Chloridoideae*, in the "PACC" clade that also contains subfamilies *Panicoideae*, *Arundinoideae*, and *Centothecoideae* (Kim et al. 2009). *Panicoideae* including maize, sorghum, sugarcane, and foxtail millet have been extensively studied, but other *Poaceae* subfamilies lag in scientific exploration. In particular, only a handful of the more

than 1,300 chloridoids have attracted genome-wide exploration, which significantly limits our understanding of their evolutionary history and our ability to exploit molecular breeding tools for their improvement. Among the four most important genera within *Chloridoideae*, *Cynodon* (including bermudagrass) and *Eleusine* (finger millet) are in the same clade (i.e., subtribe Eleusininae), *Zoysia* (zoysiagrass) is in a sister clade, and *Eragrostris* (tef) is sister to both (Peterson et al. 2010a; Soreng et al. 2015). Currently, *Cynodon* lags behind these three other chloridoid genera in genomics and molecular breeding applications.

While bermudagrass molecular marker research has shed some light on genetic diversity (Harris-Shultz et al. 2011; Kamps et al. 2011; Kang et al. 2008; Ling et al. 2012; Wu 2011; Wu et al. 2004; Wu et al. 2006; Zhiyong et al. 2013), information on structural genomics is critically low. Little is known about bermudagrass genome constitution and evolution (i.e., origin, nature of polyploidy, duplication and divergence), and chromosome behavior during meiosis (i.e., inheritance), each of which are important for evolutionary studies, genetic linkage analysis, quantitative trait loci (QTL) detection, and crop improvement. To date only three papers have reported the construction of genetic linkage maps in bermudagrass, two of which report addition of small numbers of EST-derived markers to the only available framework. The first genetic linkage framework of bermudagrass (*C. dactylon x transvaalensis*) (Bethel et al. 2006) was based on single-dose restriction fragments (SDRFs) (Wu et al. 1992) generated from probes based on bermudagrass genomic clones and heterologous cDNAs from *Pennisetum* and rice. Triploid (2n = 3x = 27) F_1 progeny were used for mapping, based on heterozygosity within each parent, following a pseudo-testcross mapping strategy (Grattapaglia and Sederoff 1994). The T89 map was based on 155 SDRFs and 17 double-dose restriction fragments (DDRFs), while that of T574 was based on 77 SDRFs. Harris et al. (2010b) added twenty-one bermudagrass-resistance gene analogs (BRGA) and EST-SSRs to the published (Bethel et al. 2006) SDRF framework. The group further updated the map with an addition of 28 EST-SSRs mined from cDNA pseudo-molecules developed at Plant Genome Mapping Laboratory (Kim et al. 2008). Further, based on disomic segregation of fourteen EST-SSRs and almost disomic profile

at yet another marker locus (single inconsistent progeny), Harris-Shultz et al. (2010a) suggested that basic chromosomes pair preferentially during meiosis and that tetraploid bermudagrass (i.e., T89) could be a segmental polyploid. Recently, Guo et al. (2015) proposed allopolyploidy of common bermudagrass based on marker segregation analysis and Gong et al. (2013) made the same proposal based on fluorescence *in situ* hybridization (FISH) and meiotic chromosome configurations.

The genome of another chloridoid, tef [*Eragrostis tef* (Zucc.) Trotter], has been sequenced (Cannarozzi et al. 2014), but due to lack of a complete and saturated linkage map, comparative genomic assessment was inadequate to delineate chromosomal rearrangements during its evolution. However, high-density sequence-tagged genetic maps of zoysiagrass (Huang et al. 2016; Wang et al. 2015a) and finger millet (Srinivasachari et al. 2007) have yielded novel insights into chloridoid genome evolution. Densely-populated sequence-tagged saturated linkage maps are needed to improve knowledge of genome structural diversity in *Poaceae* that lack genome sequences. Accordingly, a genetic linkage map in prairie cordgrass (*Spartina pectinate* Link), another chloridoid with bioenergy potential, was constructed and compared with sorghum based on homologous tef genome scaffolds (Crawford et al. 2016). Further, high-resolution genetic maps can improve genome assembly by anchoring *de novo* sequences and providing a framework to orient and order smaller scaffolds into chromosome-scale pseudomolecules (Bartholomé et al. 2015b; Fierst 2015). For example, the validity of second chloridoid genome to be sequenced and assembled (i.e., *Zoysia japonica* accession 'Nagirizaki') was authenticated and pseudo-chromosomes of the species were constructed (Tanaka et al. 2016) based on genetic linkage map from Wang et al. (2015a). However, no finished chloridoid genomes are reported; nevertheless, draft genomes are publicly available.

The development of linkage maps for many outcrossing species, including the framework linkage map of bermudagrass, relied on the "pseudo-testcross" strategy (Grattapaglia and Sederoff 1994) that involves separate estimation of parental linkage phases and recombination fractions, often using MapMaker/Exp (Lander et al. 1987). Wu et al. (2002a) developed maximum-likelihood methods for

simultaneous estimation of linkage and linkage phases, which have been successfully applied to several outcrossing species including sugarcane (Garcia et al. 2006; Oliveira et al. 2007; Palhares et al. 2012), passion fruit (Oliveira et al. 2008; Pereira et al. 2013), rubber tree (Souza et al. 2011), eucalyptus (Bartholomé et al. 2015a), and switchgrass (Lowry et al. 2015).

In the current study, application of a maximum-likelihood approach was extended to bermudagrass using functions implemented in the R software package OneMap (Margarido et al. 2007). We report the construction of complete SSR-enriched linkage maps of two bermudagrass species, *C. dactylon* x *C. transvaalensis* using the same full-sib progeny used to construct the RFLP-based framework (Bethel et al. 2006) and present an inferred ancestral chromosome complement based on alignment of the parental maps. Further, we leverage molecular approaches to assess the nature of ploidy in the tetraploid parent (i.e., T89). Additionally, we report comparative analysis with grass genome models (i.e, sorghum and rice) and four chloridoid species [i.e., tef (Cannarozzi et al. 2014), prairie cordgrass (Crawford et al. 2016), finger millet (Srinivasachari et al. 2007), and zoysiagrass (Wang et al. 2015a)] and deduce rearrangements that differentiate the modern *Cynodon* chromosomes from those of the polyploid common ancestor of Poaceae.

Materials and Methods

Mapping Population

A population of 113 F_1 hybrids from a cross between *C. dactylon* (T589; 4x) and *C. transvaalensis* (T574; 2x) developed by W. Hanna (University of Georgia, Tifton, GA) formed the basis of the framework linkage map (Bethel et al. 2006). Harris-Shultz et al. (2010a) verified diploid, triploid, and tetraploid ploidy levels of T574, the F_1s, and T89 genotypes, respectively. The two parents represent the two species crossed to produce several leading bermudagrass cultivars, including Tifgreen (Hein 1961; Robinson and Latham 1956) and Tifway (Burton 1960).

Genomic DNA and Molecular Marker Resources

Extraction of genomic DNA followed Chittenden et al. (1994) as reported by Bethel et al. (2006). In a prior study, about 2,500 simple sequence repeat (SSR) PCR primers from sugarcane expressed sequence tags (ESTs) were assayed for transferability and reproducibility in bermudagrass (our unpublished data). For a subset of about 300 markers, informative segregation and parental sources of each polymorphic band were ascertained in ten pseudo-testcross progenies. Thereafter, the presence and absence of each polymorphic band was visually determined and recorded for 95 additional F_1 individuals in the mapping population. All polymerase chain reactions (PCRs) were performed in 96-well plates, using 1µL each of MgCl2 (3 mM), dNTPs (2 mM), and 10X PCR buffer (100 mM Tris-HCL at pH 9, 500 mM KCL, and 15 mM $MgCl_2$), 0.2µL of Taq DNA polymerase (1 U), 3.8µL of ddH_2O, and 2µL of template DNA (15 ng/µL) for a total volume of 10µL. The typical cycling conditions for PCR were 95°C for 4 min in the first step, followed by 6 cycles of 94°C for 40 sec, a gradient reaction from 58°C to 52°C each for 1 min, and 72°C for 1 min. The third step was set for 35 cycles of 94°C for 40 sec, 55°C for 1 min, and 72°C for 1 min. After the last cycle, reactions were incubated at 72°C for final extension period of 10 min before cooling to 4°C. PCR amplicons were resolved by 10% nondenaturing polyacrylamide gel electrophoresis, and visualized by staining with silver nitrate (Bassam et al. 1991).

Segregation Analysis

Since different genotypic configurations are possible in segregating populations of a polyploid species, phenotype of a DNA marker is not adequate to define genotype at the underlying locus; rather, its segregation ratio across the progenies provides that information (Sorrells 1992; Wu et al. 1992). Following Wu et al. (1992), single-dose and biparental markers were tested by Chi-squared analysis for goodness of fit ($P = 0.01$) with 1:1 and 3:1 ratios expected for simplex (single copy) by nulliplex (no copy) and simplex by simplex markers, respectively. Next, parent specific double-dose (two copies) amplicons from the subgenomes, or paralogous copies of segmental duplications segregating in a 3:1 ratio were identified.

Further, tetrasomic segregation ratios for duplex (two copies) by nulliplex (5:1) and duplex by simplex (11:1) markers were also tested, following Ripol et al. (1999). Restriction fragment length polymorphism (RFLP) data for a total of 113 F_1 individuals were obtained from Bethel et al. (2006) and were pooled with SSR data for linkage analysis. Allelic SSRs and RFLPs showing disomic segregation were scored and analyzed as codominant markers. TetraploidMap (Hackett et al. 2007) was used to verify marker dosage classification and to examine significant simplex-duplex linkages.

Linkage Analysis

The analyses were carried out following procedures proposed by Wu et al. (2002b) and implemented in OneMap software (Margarido et al. 2007). First, EM algorithm-based two-point pair-wise analysis was carried out between all pairs of markers (Dempster et al. 1977), which simultaneously estimated recombination fractions and linkage phases between marker-pairs based on posterior probabilities of Bayes' theorem ('rf.2pts' command). CGs were established with a minimum LOD (logarithm of odds ratio) threshold of 4.8 and a maximum recombination fraction $\theta = 0.4$ [the maximum detectable recombination fraction under autopolyploidy in a population of 113 individuals, following Wu et al. (1992)]. Map units (cM) were calculated by the method of Kosambi (1944). Clusters of linked markers or CGs were then ordered using a step-wise approach outlined in the OneMap tutorial. In short, for CGs constituting up to 7 markers, all possible marker orders were evaluated ('compare' command) while for the groups with more than 7 markers, a subset of six to seven informative markers were ordered to build frameworks to which other markers were individually tested ('try.seq' command). Once the CGs were built, unlinked markers were tried at LODs as low as 2 ('try.seq' command). Spurious associations were detected using recombination fraction vs. LOD heatmap plots for individual CGs. The final positions of the markers were refined using ripple search (Lander et al. 1987). Instead of integrating markers that fit arbitrary orders (using 'force' command), but are significantly linked with two or more safely ordered markers within the CGs, such markers were identified as accessory markers to their closest linkages. Fragmented LGs were

identified based on weak linkages between one or more markers mapped to two or more CGs but which could not be unambiguously merged together, further supported by homology with rice and sorghum genomes. Comparative map length analyses were adjusted for the markers that were in the framework, but could not be mapped in the current study. Accordingly, two separate genetic linkage maps were built, one of each parental species.

TetraploidMap software was used to detect and verify simplex-duplex linkages in both coupling and repulsion phases and employed default Chi-squared tests with $P = 0.001$ (Hackett et al. 2007). In principle, a double dose marker (DDM) should be linked in coupling with two different CGs; ideally, these two CGs would comprise two homologous chromosomes in an autopolyploid (segregating in 5:1 ratio) or homoeologous chromosomes in an allopolyploid (segregating in 3:1 ratio). Nevertheless, a DDM could also represent a segmental duplication at non-homo(eo)logous regions (segregating in 3:1 ratio). In such cases, consistencies of identified chromosomal bridges were reconfirmed with shared multilocus probes, repulsion phase linkages, and homology with rice and sorghum genomes. Similarly, parental maps were aligned to infer ancestral chromosomes based on shared multilocus markers and biparental bridges, further supported by inferred homology with the allied plant genomes. All map charts were drawn using MapChart v2.1 (Voorrips 2002).

Marker Distribution, Estimated Recombination Length, and Genome Coverage

To examine marker distribution over the entire map, relationships between lengths (cM) of CG and numbers of markers in each CG were tested with Pearson correlation coefficients. For the estimation of genome length, two different approaches were implemented. First, the average marker spacing/interval (*s*) was calculated by dividing the total map length by the number of intervals (number of markers minus number of CGs). Then, the estimated genome length (*Ge1*) was determined by adding 2*s* to the length of each CG (Fishman et al. 2001). Second, using Method 4 of Chakravarti et al. (1991), the expected genome size (*Ge2*) was estimated as the sum of the length of each CG multiplied by (m+1)/(m-1), in which m is the

number of markers on each CG. The total map length (G_0) was the sum of the length of each CG. In order to infer monoploid estimates (monoploid defined here as a map with LGs consistent with the basal chromosome number), both methods were adjusted to account for fragmented LGs. CGs not assigned to homologous groups (HGs) were also excluded from genome size and coverage estimates for putative monoploid maps. Coverage of the *Cynodon* genome by the linkage map was estimated by the ratio G_0/Ge, where *Ge* represents the average of the estimated genome lengths from the two approaches, respectively. Genome coverage was also calculated by:

$$c = 1 - e^{-2dn/G},$$

where *c* is the proportion of the genome within *d* cM of a marker, *G* is the estimated genome length, and *n* is the number of markers in the map (Lange 1982).

Mode of Inheritance

A number of empirical approaches exist to suggest the type of inheritance (disomic/polysomic) in a polyploid species. Classical cytogenetic evidence involves meiotic chromosome pairing behavior (e.g., bivalent vs. multivalent chromosomal association) during meiosis to infer mode of inheritance (Müntzing 1936). Cytological evidence in *Cynodon* was reviewed. Further, strictly disomic segregation of codominant markers suggests preferential pairing in a segmental or a complete allopolyploid (Guo et al. 2015). Marker segregation data was scanned for codominant segregants. Third, the ratio of simplex to multiplex markers may indicate allopolyploid (0.75:0.25) or autopolyploid (0.83:0.17) genome constitution (Silva et al. 1993). Chi-squared values were estimated to test the goodness of fit to different genome constitution ratios based on observed single-dose vs. multiple dose marker frequencies. Fourth, recombination fractions and linkage phases between all marker-pairs ('rf.2pts' command) were analyzed to estimate the ratio of markers in repulsion vs. coupling phase, a measure that indicates disomic, polysomic, or intermediate modes of inheritance (Sorrells 1992; Wu et al. 1992). Within established CGs, linkage estimates were accepted at LOD thresholds as low as 2. Deviations from 1:1 ratio (i.e., equal frequency as expected in a disomic

inheritance) of repulsion vs. coupling, were tested using Chi-squared assays for individual CGs as well as at genome-wise levels (Wu et al. 1992). Statistically significant deviations suggested random chromosome pairing while non-significant deviations suggested chromosome pairing to be preferential. Fifth, linkage between putative homo(eo)logous groups would indicate either complete (autopolyploidy) or partial (segmental polyploidy) random chromosome pairing during meiosis, which could result from different levels of preferential pairing. Two-point linkage data for all marker-pairs was scanned for coupling or repulsion linkages between markers mapped to homo(eo)logous groups. Between putative homo(eo)logous groups, linkage estimates were accepted at LOD thresholds as low as 2 and $\theta = 0.4$.

Comparative Mapping

The sequences of mapped RFLP probes and ESTs corresponding to EST-SSRs were BLASTed against pseudomolecules of rice (*Oryza sativa* v 7.0) (Kawahara et al. 2013) and sorghum (*Sorghum bicolor* v2.1) (Paterson et al. 2009) using the Phytozome BLAST tool (http://phytozome.jgi.doe.gov) at a significance threshold $\leq 1 \times 10^{-10}$. The output was manually parsed to identify the most significant hits and their chromosomal locations. An ancestral chromosome consensus of bermudagrass homologs was built using MergedMap (Wu et al. 2011). Colinearity was inferred by comparing the chromosomal locations of the best rice and sorghum hits to the orders of the corresponding markers on the inferred *Cynodon* ancestral chromosomes. BLASTn (Altschul et al. 1997) implemented in Zoysia Genome Database (zoysia.kazusa.or.jp) was used to identify significant sequence homologies (at $\leq 1 \times 10^{-10}$) between bermudagrass markers and *Zoysia japonica* Nagirizaki pseudomolecules (Tanaka et al. 2016). For a comprehensive comparative assessment, studies pertinent to karyotype evolution in four chloridoid species [tef (Cannarozzi et al. 2014), prairie cordgrass (Crawford et al. 2016), finger millet (Srinivasachari et al. 2007), and zoysiagrass (Huang et al. 2016; Tanaka et al. 2016; Wang et al. 2015a)] were also compared with the current study. Chromosome nomenclature followed genus and species initials followed by

established chromosome number [i.e., *Sorghum bicolor* Sb 1 to Sb 10; *Oryza sativa* Os 1 to Os 12; *Zoysia japonica* Zj 1 to Zj 20; *Zoysia mantrella* *Zm* 1 to *Zm* 20).

Results

Segregation Analysis

A total of 116 SSR primer pairs from sugarcane ESTs amplified 240 segregating bands in bermudagrass parental samples and 105 F_1 progenies, of which 138 (57.5%) were T89-specific, 81 (33.7%) were T574-specific, and 21 (8.7%) were shared. Observed segregation ratios were calculated for a pooled dataset of 668 markers (240 SSRs and 428 RFLPs), which included a total of 21 biparental markers and 440 and 207 uniparental markers from T89 and T574, respectively. The classification of SSR and RFLP markers based on their segregation ratios is summarized in Table 3.1. In the tetraploid parent T89, 82.5% of undistorted markers were single dose and 17.5% were higher dose (P = 0.01). Interestingly, 11% of markers also satisfied double-dose ratios in the diploid parent T574 (see "Discussion"). Segregation distortion accounted for 12.1% and 9.3% of uniparental markers in T574 and T89, respectively. Three genotypes, namely B17-38, B17-39, and B17-107 showed erratic genotype patterns (for example, missing uniformly monomorphic bands, or absence of bands at codominant marker loci showing disomic segregation) for a number of markers. Since the biological basis of such occurrences were not clear (perhaps meiotic abnormalities), the three genotypes were removed from further analyses. Forty-five allelic pairs (27 T89 and 18 T574-specific) showed disomic segregation in the combined RFLP and SSR dataset, which included 14 SSR and 31 RFLP marker-pairs.

Map Construction

C. transvaalensis (2n – 2x – 18) T574 map

The T574 map was constructed based on 171 markers including 70 SSRs (50 dominant, 6 codominant, 12 biparental, and two codominant biparental) and 101 RFLPs (89 dominant and 12

codominant). A total of 125 loci were mapped with 52 SSRs (39 dominant, 6 codominant, 5 biparental, and 2 codominant biparental markers) and 73 RFLPs (61 dominant and 12 codominant) (Figure 3.1). Of a total of 46 unlinked markers, 50% deviated from the expected segregation ratios ($P = 0.05$). Based on two-point recombination fraction estimates, six unlinked markers were significantly linked (LOD ≥ 4.8, $\theta = 0.4$) with six different CGs, but they could not be unambiguously integrated into the respected CGs and were, therefore, identified as accessory markers (not shown). Three originally mapped markers were excluded from the updated map as they could not be mapped at the minimum threshold set for linkage analysis.

The integrated RFLP-SSR map is composed of 14 CGs covering a total of 1,188.4 cM, with 125 loci separated by an average of 10.7 cM (Table 3.2). On average, CGs are defined by about 9 markers and cover 84.9 cM. In comparison, the framework linkage map covered a total of 973 cM with 77 markers separated by an average of 16.5 cM and each CG was defined by about 4 markers covering about 54 cM.

Six CGs were inferred to represent fragments of three larger LGs based on weak linkages between them. T574 7a-2/b-I and T574 5a/b are two fragments of the same LG based on 4 coupling and 4 repulsion linkages supported by an average LOD of 2.42. Similarly, two novel LGs T574 11* and T574 12* with 2 and 5 markers are associated with LGs T574 9 and T574 3a/b with average linkage support of 3.69 and 5.25, respectively. As such, the final linkage map consists of 11 LGs with 2 to 18 markers each. Nine LGs with a minimum of 9 markers represented the basic chromosome complement of bermudagrass – two CGs with 2 markers each could not be associated with the major LGs (Supplemental Table 3.1).

C. dactylon (2n-4x-36) T89 map

The T89 map was constructed based on 349 markers including 139 SSRs (114 dominant, 8 codominant, 13 biparental, and 4 codominant biparental) and 210 RFLPs (191 dominant and 19 codominant). A total of 291 loci were mapped with 113 SSRs (that includes 93 dominant, 7 codominant, 9 biparental, and 4 codominant biparental) and 178 RFLPs (160 dominant and 18 codominant) (Figure 3.1). Of a total of 58 unlinked markers, 20 showed distorted segregation ratios and/or have more than 50%

missing data. Eight originally mapped markers were excluded from the updated map since they either were not mapped at the minimum threshold set for linkage analysis or were misclassified as a single dose marker (i.e., RZ557c was a DDM). Comparative map length analyses were adjusted for the missing markers. About 50% of unlinked markers were identified as accessory markers based on significant two-point recombination fractions with the linked ones (not shown).

The integrated RFLP-SSR map of T89 is composed of 34 CGs covering a total of 3,248.7 cM, with 291 marker loci separated by an average of 12.5 cM (Table 3.2). On average, CGs are defined by about 8.6 markers and cover ~ 96 cM. In comparison, the framework linkage map covered a total of 1837.3 cM, with 144 markers (136 dominant, 12 codominant) separated by an average distance of 15.3 cM and each CG was defined by about four markers and covered an average of 52.5 cM.

Based on weak linkages between the CGs, twenty-six CGs were inferred to represent 18 LGs representing 9 complete homo(eo)logous pairs – five CGs with 2 and three CGs with 3 markers could not be associated with the major LGs (Supplemental Table 3.1).

In partial summary, a total of 149 (129 dominant, 7 codominant, 9 biparental, and 4 codominant biparental) and 58 new loci (48 dominant and 10 codominant) were added to the framework linkage maps of T89 and T574, which increased Bethel et al. (2006) framework map lengths by 22% and 77%, respectively. Four and eight novel CGs, with 12 and 24 markers, together with seven and seventeen independent end-extension events (i.e., markers mapped to the ends of CGs) accounted for 71% and 100% of the increase in map lengths of T89 and T574, respectively. In comparing linkage maps, there were a few noteworthy changes to the current update. LGs T89 1a and T89 1b were split into two CGs, T89 1a/b-I and T89 1a/b-II; and LG T89 4c was also split into two homo(eo)logous CGs, T89 4c-I and T89 4c-II. Similarly, LG complex T574 7a-1, T574 7a-2, and T574 7b was split up into two LGs, namely T574 7a-2/b-I and T574 7a-1/b-II, while T574 8 and T574 10 were merged into one (with the addition of 5 new markers).

Further, LG T574 9 exclusively contained coupling linkages in the original framework, but new markers revealed several repulsion linkages.

Superimposition of double-dose markers

Bethel et al. (2006) identified 44 RFLP loci as DDRFs segregating in a 5:1 ratio in the progeny, following Ripol et al. (1999). In contrast to a complete polysomic model tested in Bethel et al. (2006), here all the markers were tested for allelic (i.e. homologous) or non-allelic (i.e., homoeologous or heterologous) duplication that is segregating in ≥ 3:1 ratio (see "Materials and methods"). A total of 74 loci (i.e., 10 SSRs and 64 RFLPs) were confirmed to have ≥ 3:1 segregation ratios in the progeny, of which 4 loci were present in T89-duplex by T574-simplex configuration (i.e., segregating in ≥ 8:1 ratio), respectively.

Out of a total of 70 informative DDMs (i.e., segregating in $3{:}1 \geq x \leq 5{:}1$), 34 (50%) DDMs were involved in a total of 77 significant simplex-duplex linkages ($P = 0.001$). Among these, 54 corresponded to DDMs linked in both coupling and repulsion to SDMs mapped to single CGs, and 10 and 13 simplex-duplex linkages were either in coupling or in repulsion within CGs, respectively. Sixteen additional DDMs showed multiple linkages with simplex markers within CGs, which developed 40 additional linkages, 30 in both coupling and repulsion and 10 only in coupling. Further, eight DDMs were linked in coupling to two different CGs. Of interest, four of the eight markers showed both coupling and repulsion linkages with CGs within assigned homo(eo)logous groups, which satisfies four possible linkages (i.e., two each of coupling and repulsion) that can be detected from a duplex marker. For example, duplex markers RZ460a and RZ557c were significantly linked (in both coupling and in repulsion) to simplex markers in CGs T89 3a/b and T89 4a/b, which formed HG 2 of the integrated map. The remaining four DDMs were linked to CGs associated with different HGs, suggesting heterologous duplications at these loci. Further, two additional DDMs were linked in both coupling and repulsion to single CGs and only in repulsion to its homo(eo)log, leaving one coupling linkage undetected. These include DDMs RZ395b and T5742B11c, which are linked

in both coupling and repulsion to T89 4c/d-II and T89 1a/b-I, while linked only in repulsion to their homo(eo)logs T89 4c/d-I and T89 2a/b, respectively.

Simplex-duplex associations with at least one significant coupling linkage were super-imposed on the integrated map (Figure 3.1), while thirteen simplex-duplex repulsion-only linkages were excluded, following Hackett et al. (2007).

Homo(eo)logy assignment of cosegregating groups

The addition of more than 200 loci to the updated map helped to clarify homologous relationships. Originally, four homologous T89 sets were aligned based on shared multi-locus probes and repulsion phase linkages (Bethel et al. 2006). For example, consensus 1 of T89 was originally established based on integration of T89 1a, T89 1b, T89 1c, and T89 1d. While several repulsion linkages were detected between T89 1a and T89 1b (i.e., T89 1a/b) and between T89 1c and T89 1d (i.e, T89 1c/d), a single repulsion linkage connected T89 1a/b with T89 1c/d. The specific simplex-simplex repulsion was also detected in this study, but the LOD support was low (< 2.5). Further, homo(eo)logy between T89 1a/b, and T89 2a/b/c garnered stronger support to build HG 9 based on 11 shared multilocus markers, 10 simplex-simplex linkages (perhaps displaying residual polysomy), 3 simplex-duplex linkages, and 11 and 14 significant blast hits with rice (i.e., Os 6 and Os 9) and sorghum (i.e., Sb 2 and Sb 10), respectively. Accordingly, nine novel HGs presumed to represent the basic chromosome number of bermudagrass (x = 9) were built and aligned, all based on strong support from shared multilocus markers, significant linkages, and homology with rice and sorghum genomes (Figure 3.1).

Estimated Recombination Lengths and Genome Coverage

The two methods implemented to estimate genome lengths (see “Materials and methods”) from the updated linkage maps assume random distribution of markers in the maps, which were tested using Pearson correlation coefficients. The coefficient values between CG lengths (in cM) and number of markers in T574 and T89 maps were 0.80 and 0.90, respectively, indicating that the marker distributions in both maps are

approximately random. Since the two estimates almost coincided, we used their average as expected genome length (Table 3.3). On the basis of the expected genome length, genome coverage for the T574 and T89 maps is 79.5% and 78.3%, respectively. Considering estimated 1C genome sizes of *C. transvaalensis* (5.43 x 10^8 bp) and *C. dactylon* (14.33 x 10^8) as cataloged in Plant DNA C-values database (http//:data.kew.org/cvalues) following Taliaferro et al. (1997), the present average interval between marker loci is equivalent to 3.9 Mbp in T574 and 4.4 Mbp in T89. Accordingly, 1 cM represents an average of 0.36 Mbp in T574 and 0.35 Mbp in T89. Both maps approach monoploidy once unassigned CGs are excluded. Since two T574 and eight T89 CGs have fewer than 4 markers ($m < 4$) and remain unassigned to HGs, they were excluded from marker distribution and genome size and coverage estimates. When only the monoploid maps are taken into consideration, genome coverage increases to 85.8% in T574 and 87.0% in T89, with 4.3 and 5.1 Mbp marker intervals and 0.39 and 0.40 Mbp per cM, respectively. For both maps, ~80% of the genome was within 10 cM of a mapped marker.

Compared to the framework linkage map, updated genome size estimate decreased the T574 map by 73.5 cM and increased the T89 map by 1,140.2 cM. However, genome coverage increased by ~ 17% in both species.

Mode of Inheritance in T89

Codominant markers and disomic segregation

A total of 27 T89-specific codominant markers including 8 pairs of SSR and 19 pairs of RFLP alleles were detected, which showed strict disomic segregation at parental heterozygous loci (i.e. 100% complementary presence or absence between the two alleles). Twenty-five codominant markers were associated with 14 different CGs and correspond to 6 HGs, while 2 codominant markers remain unlinked (Figure 3.1).

Single dose vs. multiple dose markers

SSR markers showed significant deviations (P = 0.01) from the expected ratios of 3:1 and 5:1 (single dose vs. multiple dose marker frequencies) for allopolyploid and autopolyploid genome constitution, respectively (Table 3.4). Dosage ratios for RFLP markers did not deviate significantly from the allopolyploid ratio, while that for overall marker data did not deviate significantly from 5:1 ratio, indicating a much closer correspondence with autopolyploidy.

Frequency of repulsion-phase vs. coupling-phase

A total of 687 coupling and 595 repulsion linkages (LOD ≥ 2, θ = 0.4) were detected with averages of 20.2 coupling and 17.5 repulsion per CG (Supplemental Table 3.2). Although a Chi-squared test determined that the genome-wide ratio of repulsion to coupling linkage was significantly different from a disomic model (i.e. 1:1; P = 0.05), 20 of 26 CGs did not deviate significantly from the disomic preferential pairing ratio, while eight unassigned CGs with less than 4 markers (m < 4) were not tested. Further, repulsion linkages were detected across many CGs at linkages of LOD ≥ 4 and θ = 0.33, which would be highly unlikely under a completely polysomic model of inheritance (see "Discussion"). The 6 CGs showing significant divergence from the preferential pairing ratio included T89 4c-1/d, and T89 4c-2/d, the homo(eo)logous pair constituting HG 1; T89 CGs constituting HG 8; and T89 11 and T89 6a/b.

Linkages between homo(eo)logous groups

Four homo(eo)logous CG pairs shared linkages within themselves (i.e., markers in putative homoeologs show significant linkages or pseudolinkages) which is suggestive that these homo(eo)logs show incomplete, but random pairing during meiosis (i.e., residual polysomic inheritance). Homo(eo)logous LGs T89 4c/d-I and T89 4c/d-II share seven repulsion and six coupling linkages among 11 simplex markers with average recombination fraction of 0.26 each, supported by average LODs of 3.4 and 2.8, respectively. One significant repulsion phase duplex-simplex linkage was also detected between the two groups. Similarly, homo(eo)logous pair T89 1c/d and T89 7a/b share a coupling linkage (with

recombination fraction r = 0.26 and LOD = 3), while T89 13 and T89 19 share a repulsion linkage (with r = 0.31 and LOD = 3.3). Further, homo(eo)logous LG pairs T89 2a/b/c and T89 1a/b share six repulsion and four coupling linkages with average recombination fraction of 0.26 and 0.25, respectively, and supported by average LOD of 3.5. Three double-dose markers were also significantly linked to the two LG pairs (T89 2a/b/c and T89 1a/b), supporting the veracity of homo(eo)logy assignment.

Comparative Mapping

A bermudagrass ancestral chromosome complement was inferred (see "Materials and methods") and compared with sorghum and rice genomes to identify significant homologies and colinearity (Figure 3.1). Five and four of the nine bermudagrass consensus groups corresponded to single homologous chromosomes of sorghum and rice, respectively (Table 3.5). Each of the remaining four and five inferred consensus groups corresponded to two different chromosomes of sorghum and rice, respectively. Interchromosomal rearrangements were evident in HG 5 and HG 9 which involved parts of sorghum chromosomes Sb 2 and Sb 1 relocated into Sb 10 and Sb 4, respectively. These structural rearrangements were also evident when compared to cognate rice homologs and involved nested chromosome fusions (NCFs) between Os 2 and Os 10 and between Os 6 and Os 9.

Inferred ancestral chromosomes were also compared with zoysiagrass pseudomolecules (Tanaka et al. 2016) based on ninety-nine putative homologous sequences (Table 3.5). Comparative mapping showed that two major NCFs observed in bermudagrass (i.e., in HG 5 and HG 9) were also shared by zoysiagrass. These NCFs were also reported in prior studies in zoysiagrass (Huang et al. 2016; Wang et al. 2015a). Specifically, middle section of Sb 1 nested within Sb 4 in inferred ancestral HG 5 of bermudagrass was consistent with middle section of Sb 1 associated with middle sections of zoysiagrass homologs Zj/Zm 7 and Zj/Zm 8. Similarly, middle section of Sb 2 nested within Sb 10 in inferred ancestral HG 9 was consistent with middle section of Sb 2 corresponding to middle sections of Zj/Zm 19 and Zj/Zm 20. The two NCFs also corroborate with translocation-mediated evolution of finger millet group 2 chromosomes (2A and 2B)

and group 6 chromosomes (6A and 6b) as reported (Srinivasachari et al. 2007). Corresponding rearrangements has not been specified in studies concerning other two chloridoids [i.e., tef (Cannarozzi et al. 2014) and prairie cordgrass (Crawford et al. 2016)]. In tef, draft genome assembly was anchored against sorghum pseudomolecules, but a lack of complete and saturated genetic linkage map hindered clear delineation of its karyotype evolution. In prairie cordgrass, genetic markers were anchored to tef scaffolds, which served as a bridge to align its linkage maps to sorghum. Nevertheless, comparative plots reported in tef and prairie cordgrass displayed correspondences relevant to the current study. For example, correspondence chart between tef genetic map (Zeid et al. 2010) and its pseudo-chromosomes apparently traced marker(s) in LG 10 to pseudo-chromosomes 2 and 10 and 11 to 1 and 4 (Cannarozzi et al. 2014). Similarly, prairie cordgrass linkage group 6 corresponded to Sb 1 and Sb 4, while linkage group 4 corresponded to Sb2 and Sb 10. These observations support our assertion that two shared NCFs delineate karyotype evolution in the Chloridoideae subfamily and are distinct from those defining divergent Poales lineages (see "Discussion").

Two additional genome repatterning events were observed in bermudagrass. Several markers in HG 8 showed significant correspondence with Os 11 and Os 12 in the rice genome (corresponding to sorghum Sb 5 and Sb 8). Second, HG 2 showed significant correspondence with both Os 4 and Os 3 (corresponding to Sb 6 and Sb 1), which may represent yet another major reshuffling in the bermudagrass lineage.

Discussion

We report SSR-enriched bermudagrass genetic linkage maps constructed using a full-sib (i.e., F_1) population derived from an interspecific cross between two heterozygous accessions representing tetraploid common bermudagrass (i.e., T89) and diploid African bermudagrass (i.e., T574) as female and male, respectively. Clonal selections from the crosses between these two species have also produced several leading turf varieties, which underscores the importance of investigation into their genomic organization

and chromosomal transmission. Further, perenniality and the clonal nature of the mapping population provides a unique opportunity for QTL analyses across different environments over several years. The information presented in this study establishes a foundation both for basic and applied genetic studies.

The current map adds 207 novel loci to the parental linkage frameworks published earlier (Bethel et al. 2006), which increased genome coverage by ~17% to represent around 85% of the monoploid estimates of both species. In a comparable study, genome coverage with 261 and 303 markers in *Miscanthus* (*M. sacchariflorus* Robustus x *M. sinensis*), was 72.7 and 84.9%, respectively (Kim et al. 2012). Further, the number of SSR polymorphisms that fell on tetraploid chromosomes (i.e., T89) was almost exactly twice that of the diploid (i.e., T574), which is identical to RFLP estimates in Bethel et al. (2006). Therefore, considering more than two-fold differences in monoploid length estimates between the diploid and the tetraploid, heterozygosity within T574 and T89 appear comparable.

In the updated parental linkage maps, coupling and repulsion linkages were integrated. However, marker orders did not change substantially relative to the orders in the framework linkage maps (Bethel et al. 2006), supporting correspondence between the results from EM algorithm employed in OneMap (Wu et al. 2002) and the traditional "pseudo-testcross" strategy (Grattapaglia and Sederoff 1994) using MapMaker/EXP (Lander et al. 1987). Since OneMap did not support integrating duplex markers into simplex linkage maps despite established statistical basis (da Silva et al. 1995; Ripol et al. 1999), we used TetraploidMap (Hackett et al. 2007) to detect and verify duplex-simplex linkages. Most duplex-simplex linkages detected and superimposed on the original framework (Bethel et al. 2006) were also verified with TetraploidMap. Fragmented LGs were evident, moreso in T89 than T574. Support for some of the fragmented LGs (e.g., HG3 and HG5) was clearly provided by comparative mapping, but more markers are needed to join CGs into complete LGs. For example, CGs of T89 and T574 constituting HG 3 were fragmented, but showed significant BLAST homologies with two different clusters of DNA sequences separated by around 50 Mb in Sb 3 and ~ 21 Mb in Os 1 (Figure 3.1). Since codominant markers are more

informative and reliable than random dominant markers (Mollinari et al. 2009; Wu et al. 2003), future mapping efforts will target codominant markers, preferably from bermudagrass sequences homologous to current gaps in coverage of sorghum and rice genomes.

In a pseudo-testcross mapping scheme, biparental markers are necessary to integrate parent-specific linkage maps into a consensus framework (Grattapaglia and Sederoff 1994). In the current study, most SSR markers (84.5% of total) did not deviate significantly from the 1:1 ratio (i.e., presence vs. absence) expected for a single dose segregant in a testcross configuration [i.e., simplex (heterozygous in one parent) by nulliplex (recessive homozygous for null alleles in another parent)], while few SSRs (5.4%) fit the 3:1 ratio (presence vs. absence) expected for a biparental marker in an intercross configuration (i.e., double-simplex or heterozygous in both parents). As such, separate parental linkage maps were constructed due to a paucity of informative bridges. Therefore, more informative markers are needed [i.e., biparental or symmetric (ao x ao) markers] that are closely linked with partially informative ones [(e.g., asymmetric dominant (ao x oo)], where 'a' represents presence of an allele and 'o', a null allele, following (Wu et al. 2002). However, a preponderance of single dose markers and relative paucity of intercross markers are generally expected in interspecific pseudo-testcross mapping. For example, interspecific crosses between *Miscanthus sinensis* x *M. sacchariflorus* (Kim et al. 2012), *Coffea liberica* x *C. eugenioides* (Gartner et al. 2013), and *Poplar adenopoda* x *P. alba* (Yin et al. 2001) each segregated for ~ 2% of intercross markers. Since the frequency of intercross markers can be significantly improved with intraspecific mapping populations, *C. dactylon* intraspecies maps would provide better opportunities for an integrated map. For example, intraspecific crosses of *Miscanthus sinensis* (Atienza et al. 2002), *Lolium multiflorum* (Hirata et al. 2006), *Fagus sylvatica* (Scalfi et al. 2004), and *Populus deltoides* (Wu et al. 2000), provided 51.5%, 36%, 15%, and 15% of intercross markers, respectively.

Double-dose markers were inferred by a Chi-squared test based on non-significant deviation from ≥ 3:1 ratios with 99% confidence, which are expected in allelic segregation (i.e., 5:1 ratio between

homologous alleles) and non-allelic assortment (i.e., 3:1 ratio between homoeologous or paralogous loci) during tetrasomic (random chromosome pairing) and disomic (preferential chromosome pairing) inheritance, respectively. Interestingly, 17 T574 markers also fit ≥ 3:1 ratios, 7 of which were associated with CGs T574 1a/b and 3a/b. Presence of double-dose markers in diploid *C. transvaalensis* may come from illegitimate recombination, suggested by the observation of Forbes and Burton (1963) of irregular meiosis in some accessions of the species. We note that these chromosomes do not appear to be ancient homoeologs.

Mode of Inheritance

Polyploidy creates evolutionary novelty (Soltis et al. 2014). The ubiquity of polyploid species in higher plant taxa and their recurrent origins assert to their evolutionarily success (Soltis and Soltis 1999). The age and degree of homology between subgenomes of a polyploid influences its chromosomal pairing behavior at meiosis, which dictates its mode of inheritance (i.e., disomic or polysomic). Polysomic inheritance through random chromosome pairing is associated with autopolyploidy (duplication of a single genome), while disomic inheritance through preferential chromosome pairing is associated with allopolyploidy (merger of divergent genomes in a common nucleus by interspecific hybridization). In autopolyploids, 'diploidization', comprising divergence of homologous sequences and chromosomes by a variety of mechanisms, leads to a shift from random to preferential chromosome pairing; consequently, an autopolyploid may eventually become a diploid species (DeWet 1980; Le Comber et al. 2010). As such, species of autopolyploid origin may display random pairing among some homologs and preferential pairing among others during meiosis (Lentz et al. 1983; Matsubayashi 1991; Sybenga 1994), leading to a mosaic of disomic and polysomic inheritance at different loci, and making it difficult to unambiguously determine genome constitution (Stebbins 1947).

In the current study, we assessed different lines of evidence regarding the type of polyploidy in tetraploid bermudagrass, for which long standing classification as an autopolyploid (Harlan et al. 1966;

Hoff 1968) has recently been challenged (Gong et al. 2013; Guo et al. 2015; Harris-Shultz et al. 2010a). Cytological studies in tetraploid accessions of common bermudagrass frequently report multivalents (Forbes and Burton 1963, Hanna and Burton 1977, Hoff 1968), which may suggest autopolyploid genome constitution of the cosmopolitan species (Muntzing 1936). However, a low frequency of multivalents and possibility of subgenome differentiation in tetraploid *C. dactylon* may either indicate segmental allopolyploidy or almost complete diploidization following its autopolyploid origin (Brilman 1981). Fluorescence *in situ* hybridization and meiotic study by Gong et al. (2013) further reinforces the notion. Notably, the proposed genome constitution of tetraploid bermudagrass has not been revisited and unequivocally reviewed. Nevertheless, the occurrence of a high frequency of functional unreduced gametes (Harlan and de Wet 1969) and presence of both diploid and tetraploid chromosome races in at least three different *Cynodon* species (de Wet and Harlan 1970) provide a cytological basis for autopolyploid origin of tetraploid accessions. Further, a single extant diploid species (i.e., *C dactylon* var. *aridus*) shares similar morphological features with common bermudagrass (i.e., *C. dactylon* var. dactylon), including the presence of rhizomes, and displays cross compatibility and sympatric distribution with the latter, suggesting that the diploid species is the only likely source of the tetraploid accession (Harlan and de Wet 1969, Wu 2011). However, the current state of ploidy cannot be unambiguously ascertained solely based on cytotaxonomic and/or morphotaxonomic studies.

Bethel et al. (2006), Harris-Shultz et al. (2010a), and Guo et al. (2015) have used molecular markers to decipher the genome constitution of common bermudagrass. In this study, detection of 25 pairs of T89-specific alleles showing disomic inheritance in the segregating progenies suggest preferential pairing among 14 CGs carrying those alleles. Harris-Shultz et al. (2010a) also found 14 codominant EST-SSRs showing disomic inheritance in the same population. The results are also consistent with Guo et al. (2015), in which 13 of 32 codominant SSRs did not deviate significantly from the 1:2:1 ratio ($P = 0.01$) expected in selfed progenies (S1) of a heterozygous individual. These studies all indicate subgenome differentiation, strong preferential pairing, and disomic inheritance - attributes which would suggest allopolyploidy as the

current state of genome constitution in common bermudagrass. However, marker profiles for 18 primer pairs in Guo et al. (2015) did not fit either disomic or polysomic inheritance ratios (although smaller χ^2 values were associated with disomic ratios), while one locus did not deviate significantly from either (as tested by the authors). Similarly, akin to three genotypes showing uncharacteristic segregation patterns in this study (see "Results"), Harris-Shultz et al. (2010a) also found one aberrant genotype with null alleles at a codominant locus, which could be because of meiotic aberration [as frequently reported in tetraploid accessions (Forbes and Burton 1963, Hanna and Burton 1977, Hoff et al. 1968)] or incomplete preferential pairing (i.e., a characteristic of a segmental polyploid). Further, three homo(eo)logous pairs in our study lacked codominant markers and displayed partial polysomic inheritance (further discussed below). As such, common bermudagrass apparently shows mixed inheritance, a hypothesis that may be further supported by the fact that the ratio of simplex to multiplex markers for RFLPs did not deviate significantly from the disomic ratio, while that for overall marker data fit polysomic expectations. However, different marker systems (i.e., RFLPs and SSRs) may show different levels of subgenome differentiation, with SSRs being well known to evolve new alleles relatively rapidly. Accordingly, ratios of simplex to multiplex markers could be characteristically different between the two marker systems and warrant further investigation.

Comparable numbers of markers in repulsion and coupling linkages for 20 of 26 testable co-segregating groups suggest that most basic *Cynodon* chromosomes pair preferentially. Further, the majority of significant repulsion linkages were detected at $\theta = 0.33$, which would be unlikely under a complete polysomic model of inheritance. Even completely linked markers in repulsion phase (i.e., $Rc = 0$, where Rc is recombination fraction due to crossing-over) would show a recombination fraction of 0.33 due to independent assortment alone (i.e., $Ri = 0.33$, where Ri recombination fraction resulting from independent assortment) (Qu and Hancock 2001). In fact, repulsion linkages associated with six CGs that did not fit the disomic model of inheritance were also detected at $\theta = 0.33$, which would strongly suggest partial preferential association rather than completely random homologous pairing during meiosis. Further, four of these six CGs were also associated with two homo(eo)logous pairs that share linkages. In particular,

homo(eo)logs T89 4c/d-I and T89 4c/d-II shared 13 and 1 significant simplex-simplex and simplex-duplex linkages, respectively, while homo(eo)logs T89 13 and T89 19 shared a repulsion phase marker. These lines of evidence together with a lack of codominant markers suggest that these homo(eo)logous groups exhibit partial polysomic inheritance. In the past, unequivocal cases of segmental polyploidy were lacking (Sybenga 1996), but recently, cases of residual polysomy and/or intermediate inheritance have been reported in several plant species including octoploid strawberry (Rousseau-Gueutin et al. 2008) and garden dahlias (Schie et al. 2014), hexaploid chrysanthemum (Linde et al. 2014) and *Pennisetum*, tetraploid buffelgrass (Jessup et al. 2003) and yellow cress (Stift et al. 2008).

Based on the results of the current studies and long standing evidence that tetraploid bermudagrass has an autopolyploid origin, we conclude that it is in a relatively advanced state of diploidization, behaving as a segmental polyploid. As Wu et al. (1992) put it in their seminal paper: "polyploidy is a state, not a process or an event". Tetraploid bermudagrass appears to represent a species in the process of transitioning to a diploid state. The transition is further evident from the facts that very few multilocus markers mapped to the homo(eo)logous CGs and that several homo(eo)logous pairs showed rearrangements. Further, while almost twice as many polymorphisms occurred in T89 than T574, ploidy-independent frequency of amplicons was observed between the two parental accessions (our unpublished data), indicating that primer pairs predominantly amplified only one of the two homo(eo)logous loci in T89. These lines of evidence strongly suggest substantial subgenome differentiation in common bermudagrass, which is further supported by Guo et al. (2015), where only one primer pair (out of thirty-three) potentially amplified homo(eo)logous amplicons. Also, organellar and nuclear DNA evidence suggested post-WGD rapid diversification of polyploid bermudagrass (Gulsen et al. 2009; Gulsen 2011). However, high-density genetic linkage maps, preferably using third-generation marker systems (i.e., genotyping-by-sequencing), are warranted to clearly delineate subgenome differentiation in common bermudagrass.

Genome Evolution in Chloridoid Lineages

Grasses evolved from a base ancestral karyotype of *n* =7 that subsequently underwent WGD (*n*=14) followed by two chromosome fusions (CFs) to attain an intermediate ancestral karyotype of *n*=12, which among extant grasses is best represented by the modern rice (*n*=12) genome (Wang et al. 2015b). Comparative analysis indicated one-to-one and one-to-two correspondence between bermudagrass inferred ancestral chromosomes and cognate sorghum and rice chromosomes (Table 3.5). General conservation of synteny and colinearity was evident between bermudagrass and the two model genomes, with few large-scale chromosomal rearrangements. Two NCFs in bermudagrass (i.e., in HG 5 and HG 9) were also detected in zoysiagrass (n = 10) (Table 3.5) (Huang et al. 2016; Wang et al. 2015a) and finger millet (n = 9) (Srinivasachari et al. 2007), while tef and prairie cordgrass genomes also apparently share these rearrangements (see "Results"), which implicates these NCFs in the 14-to-10 chromosomal reduction process in chloridoid lineages. Therefore, they may represent major genome restructuring, perhaps in a single step, in the common ancestral chloridoid. Evidently, these NCFs are different from those that occurred in sorghum and foxtail millet (Murat et al. 2010; Wang et al. 2015b). Furthermore, bermudagrass and finger millet underwent additional genome reshuffling that conferred their basal chromosome number of 9. Two additional genome repatterning events were evident in bermudagrass, which were both different from the third NCF event in the finger millet lineage involving Sb 8 and Sb 9 (corresponding to Os 12 and Os 5) (Srinivasachari et al. 2007). Instead, the third probable NCF event in bermudagrass may involve a merger between paleo-homoeologous chromosomes Sb 5 and Sb 8 (corresponding to Os 11 and Os 12), to constitute HG 8. Akin to *Brachypodium* in which three of 7 NCF events involved ancestral duplications (Wang et al. 2015b), bermudagrass HG 8 may represent a fusion between the two pan-cereal paleo-homoeologs.

Similarly, HG 2 showed significant homology with Sb 6 and Sb 1 (corresponding to Os 4 and Os 3), which may represent yet another major reshuffling in the bermudagrass lineage (a hypothesis that needs

further assessment). Although the tef genome would provide additional support for comparative analysis in chloridoid species, lack of complete and saturated linkage maps curtailed the possibility of aligning its pseudo-chromosomes to its linkage maps to adequately delineate its chromosomal rearrangements (Cannarozzi et al. 2014). A tef-specific translocation between Sb 3 and Sb 9 (Cannarozzi et al. 2014) was evidently not shared by zoysiagrass (Huang et al. 2016; Tanaka et al. 2016; Wang et al. 2015a), finger millet (Srinivasachari et al. 2007) or bermudagrass (current study), suggesting species-specific rearrangement. Although the current study did not permit large scale comparative analysis to make unequivocal assertions about bermudagrass genome evolution, it demonstrates the feasibility of comparative genomics to navigate inadequately charted genomic landscapes of the chloridoids.

Conclusion

In the current study, a framework linkage map of the bermudagrass genome based on an interspecific cross (*C. dactylon* x *transvaalensis*) was substantially enriched, providing new insight into chloridoid transmission and evolutionary genetics. Common bermudagrass primarily showed disomic inheritance with irrefutable hints of residual polysomic inheritance, indicating that its current genome constitution is a segmental allopolyploid. The updated map is suitable for exploratory QTL analysis since ~80% of the genome lies within 10 cM of a mapped marker. Accordingly, we are currently investigating marker-trait associations for a number of qualitative and quantitative traits relevant to the bermudagrass industry, particularly turf. Basic and applied genetic research in bermudagrass is expected to broaden understanding of evolutionary, structural, and functional genomics of plants.

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Table 3.1 Classification of marker inheritance in bermudagrass based on Chi-squared assays ($P = 0.01$), following Wu, Burnquist, et al. (1992)

Marker/classification	*C. transvaalensis* (T574)	*C. dactylon* (T89)	Bi-parental	Total
SSRs				
Single dose (1:1)	60	118		178
Biparental (3:1)			13	13
Double dose (3:1) or (5:1)		6		6
Double dose (8:1) or (11:1)			4	4
Segregation distorted	21	14	4	39
RFLPs [a]				
Single dose (1:1)	102	211		313
Double dose (3:1) or (5:1)	20	64		84
Segregation distorted	4	27		31
Total	207	440	21	668

[a] RFLPs were developed and used by Bethel, Sciara, et al. (2006)

Table 3.2 Comparison of T574 and T89 genetic linkage maps of *Cynodon* spp.

	C. transvaalensis (T574)	*C. dactylon* (T89)
Number of cosegregating groups (CGs)	14	34
Total map length (cM)	1188.4	3248.7
Mean length of CG (cM)	84.9	96.0
Largest size of CG (cM)	161.4	233.4
Smallest size of CG (cM)	3.2	4.5
Total number of markers	125	291
RFLP markers	73	178
Codominant	12	18
Dominant	61	160
SSR markers	52	113
Codominant	6	7
Dominant	39	93
Biparental	5	9
Codominant biparental	2	4
Average distance between markers (cM)	10.7	12.5
Marker density (map length/number of markers)	9.5	11.2
Average number of markers per CG	9	8.6
Largest gap size (cM)	29.7	37.8
Number of gaps >30 cM	0	6

Table 3.3 Expected and observed map length and genome coverage of *C. transvaalensis* (T574) and *C. dactylon* (T89) genetic maps

	T574	T89	T574 Monoploid-map	T89 Monoploid-map
G_{e1} (cM)	1,488.3	4,076.5	1,373.6	3,563.0
G_{e2} (cM)	1,502.7	4,227.9	1,378.4	3,578.7
G_e (cM)	1,495.5	4,152.2	1,376.0	3,570.8
G_O (cM)	1,188.4	3,249.6	1,181.1	3,105.8
Genome coverage (%)	79.5	78.3	85.8	87.0
1C genome size (Mbp)	543	1,433	543	1,433
Genome coverage (Mbp)	431	1,121	466	1,246
Mbp per marker interval	3.9	4.4	4.3	5.1
Mbp per cM	0.36	0.35	0.39	0.40

T574 and T89 monoploid maps; from HG 1 to HG 9. 1C genome size was obtained from Taliaferro, Hopkins, et al. (1997). *Mbp* mega base pair, G_{e1} estimated length by Fishman et al. (2001), G_{e2} estimated length by Chakravarti et al. (1991), G_e average of G_{e1} and G_{e2}, G_o observed length

Table 3.4 Summary of Chi-squared tests of simplex to multiplex ratios of T89-associated markers compared to theoretical expectations in allopolyploids and autopolyploids

Marker	Observed (in T89)	Allopolyploid		Autopolyploid	
		Expected	χ^2	Expected	χ^2
SSR					
Simplex	131	105.8	24.11**	117.0	9.81**
Multiplex	10	35.3		24.0	
	141	141		141	
RFLP					
Simplex	211	206.3	0.44 *ns*	228.3	7.67**
Multiplex	64	68.8		46.8	
	275	275		275	
Total					
Simplex	342	312.0	11.54**	345.3	0.18 *ns*
Multiplex	74	104.0		70.7	
	416	416		416	

**Significant at $P = 0.01$; *ns* not significant

Table 3.5 Number of markers that share high degrees of similarity with sorghum, rice and zoysiagrass pseudomolecules

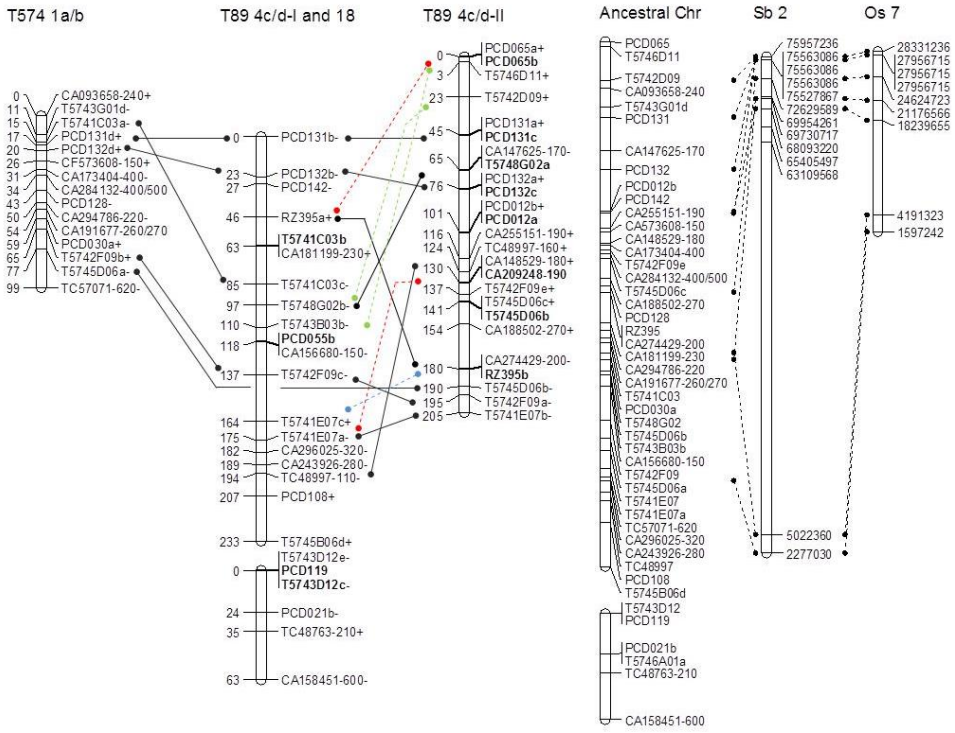
Homologous Group (HG)	Orthologous sorghum chromosome	Significant hits to sorghum genome [a]	Orthologous rice chromosome	Significant hits to rice genome	Orthologous zoysiagrass chromosomes (homoeologs)	Significant hits to zoysiagrass genome [b]
HG 1	Sb 2	13	Os 7	9	Zj 3 and Zj 4	3 and 6 (9)
HG 2	Sb 1 + Sb 6	8 + 7 (15)	Os 3 + Os 4	4 + 4 (8)	Zj 11 and Zj 12	5 and 6 (11)
HG 3	Sb 3	11	Os 1 + Os 12	9 + 5 (14)	Zj 5 + Zj 15 and Zj 6 + Zj 16	7 + 0 and 6 + 0 (13)
HG 4	Sb 7	11	Os 8	7	Zj 13 and Zj 14	3 and 3 (6)
HG 5	Sb 4 + Sb1	11 + 7 (18)	Os 2 + Os 10	11 + 5 (16)	Zj 7 + Zj 1 and Zj 8 + Zj 2	12 + 3 and 12 + 3 (30)
HG 6	Sb 1	11	Os 3	12	Zj 1 and Zj 2	4 and 4 (8)
HG 7	Sb 9	6	Os 5	5	Zj 17 and Zj 18	3 and 3 (6)
HG 8	Sb 5 + Sb 8	3 + 5 (8)	Os 11 + Os 12	3 + 4 (7)	Zj 9 + Zj 15 and Zj 10 + Zj16	0 + 1 and 2 + 1 (4)
HG 9	Sb 10 + Sb 2	6 + 9 (15)	Os 6 + Os 9	7 + 5 (12)	Zj 19 and Zj 20	6 and 6 (12)
Total		108		91		

[a] Numbers in parenthesis are sums of significant hits to different chromosomes

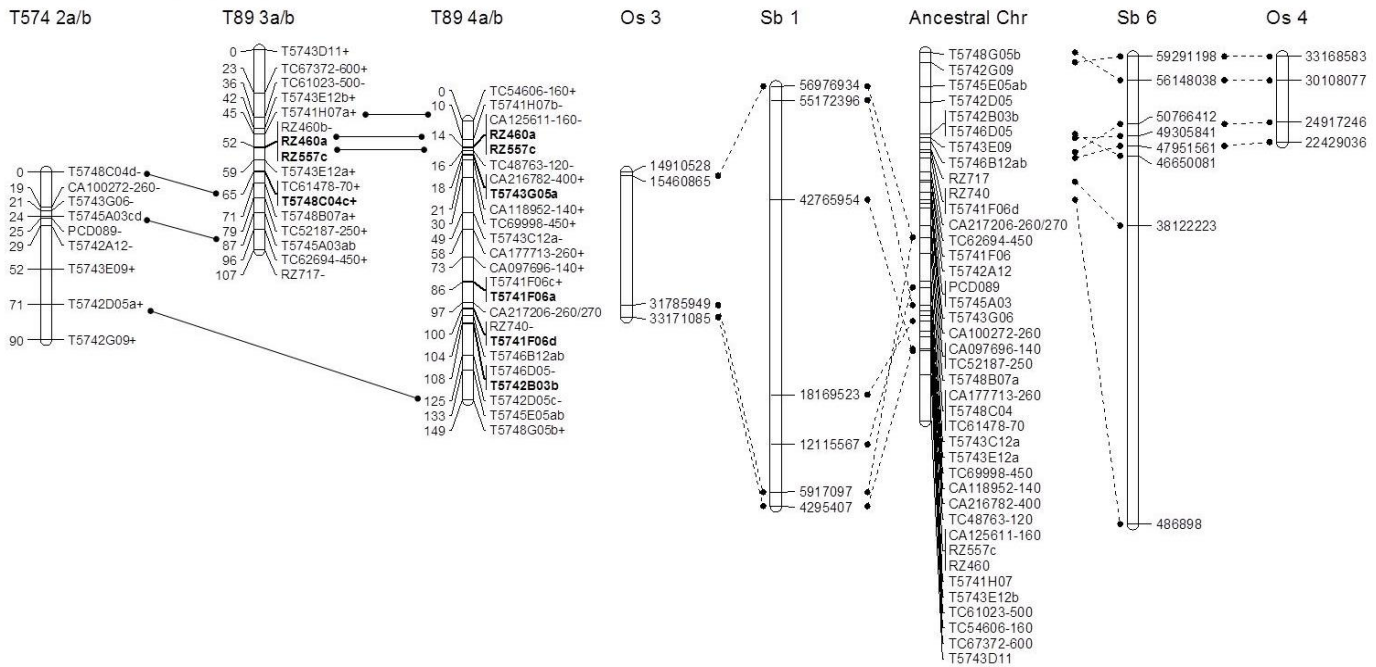
[b] Numbers correspond to significant hits to homoeologous chromosomes and their sums in parenthesis

Figure 3.1 Linkage maps of *Cynodon* spp., inferred ancestral bermudagrass chromosomes, and homology with *Sorghum* and rice. *Cynodon transvaalensis* (2x = 18) and *C. dactylon* (4x = 36) CGs are identified with *T574* and *T89* prefixes, while inferred bermudagrass ancestral chromosomes between the two parents are compared with *Sorghum* (*Sb*) and rice (*Os*) homologs. CA-, CF-, and TC-type markers are sugarcane EST-SSRs. The '±' suffix to mapped markers designates alternative linkage phases. Markers covered by vertical lines map to same locus or in case of a DDM (*bold font*), represents closest linkages. Markers are indicated on the *right*, cumulative distances are on the *left* (in Kosambi cM). T89 map consists of 34 CGs, of which 26 CGs are assigned to one of nine homologous groups. Likewise, twelve of fourteen T574 CGs are assigned to homolgous groups. T89 homo(eo)logs are connected with shared multi-locus markers (*solid lines*) and simplex-simplex (*green-dashed* coupling; *red-dashed* repulsion) and simplex-duplex coupling linkages (*blue-dashed* lines). Homologous groups between T574 and T89 are aligned on the basis of multi-locus markers including biparental loci (*solid lines*). *Dashed-solid black lines* connect inferred bermudagrass ancestral chromosomes to putative *Sorghum* and rice homologs.

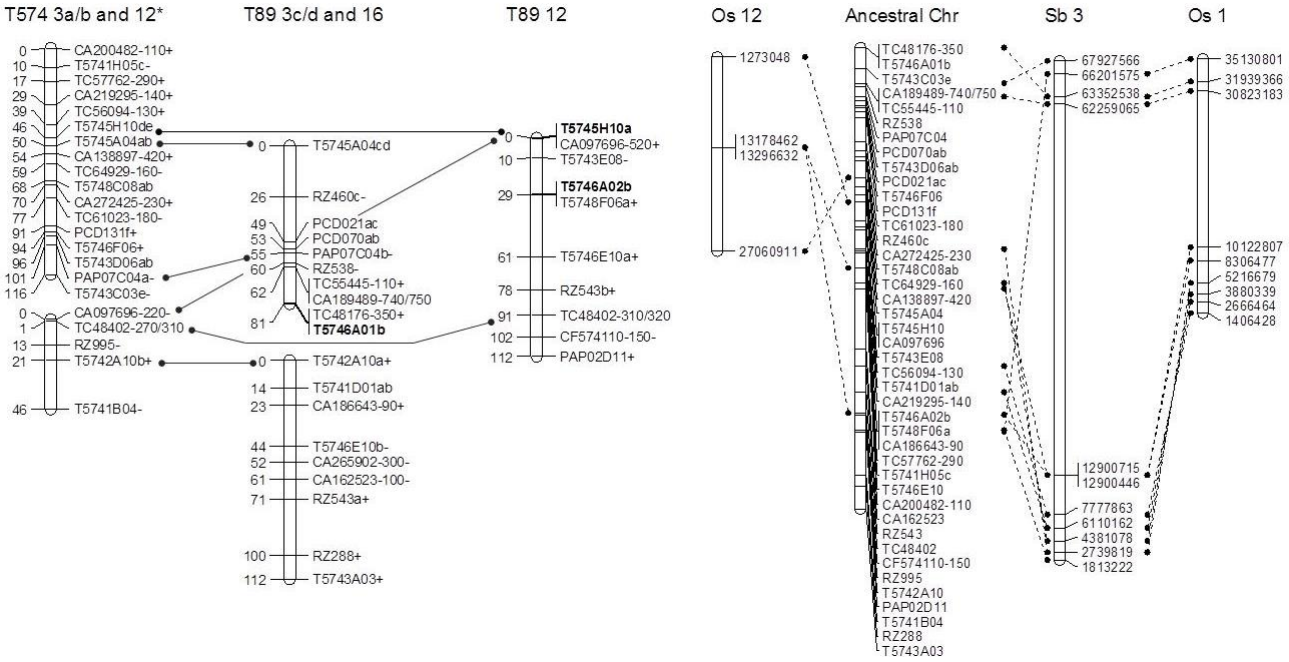
Homologous Group 1 (HG 1)



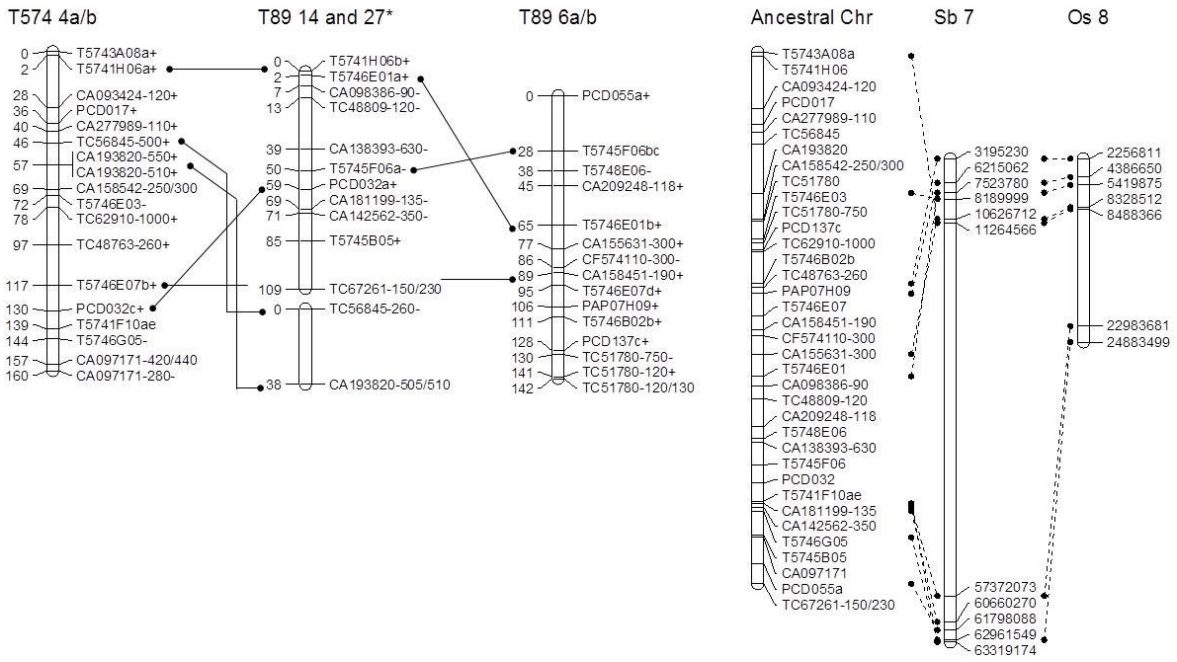
Homologous Group 2 (HG 2)



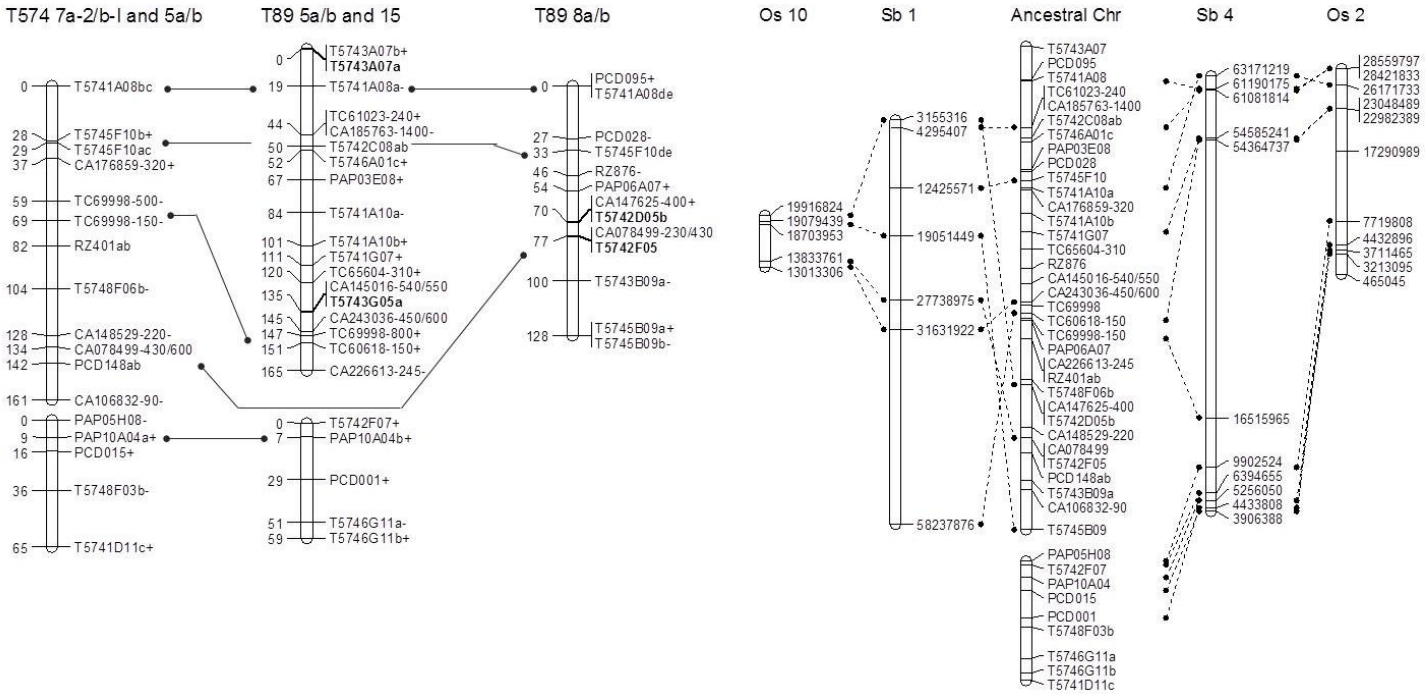
Homologous Group 3 (HG 3)



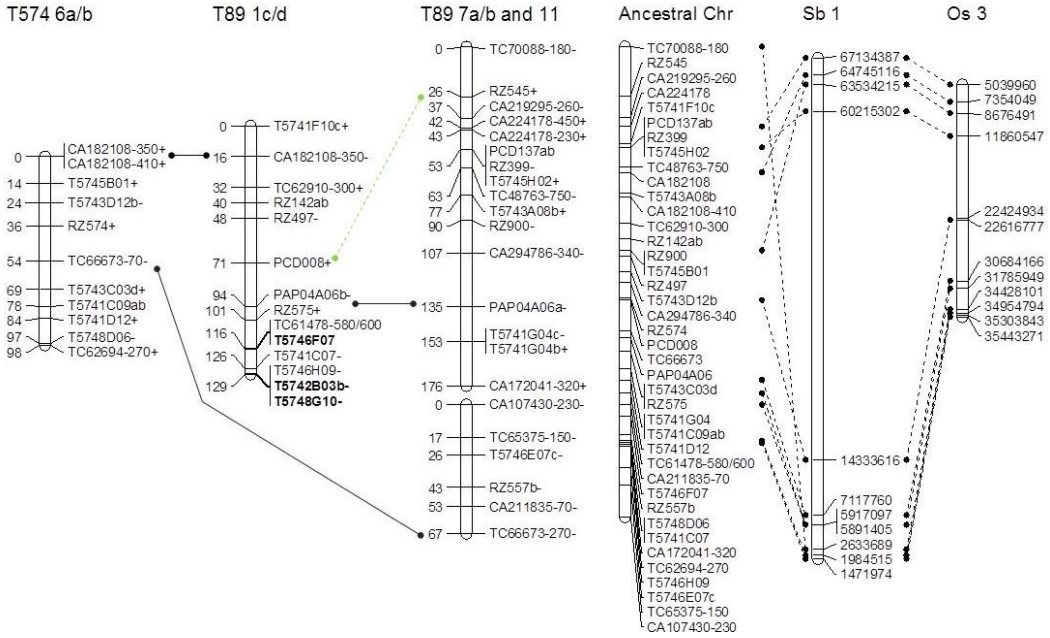
Homologous Group 4 (HG 4)



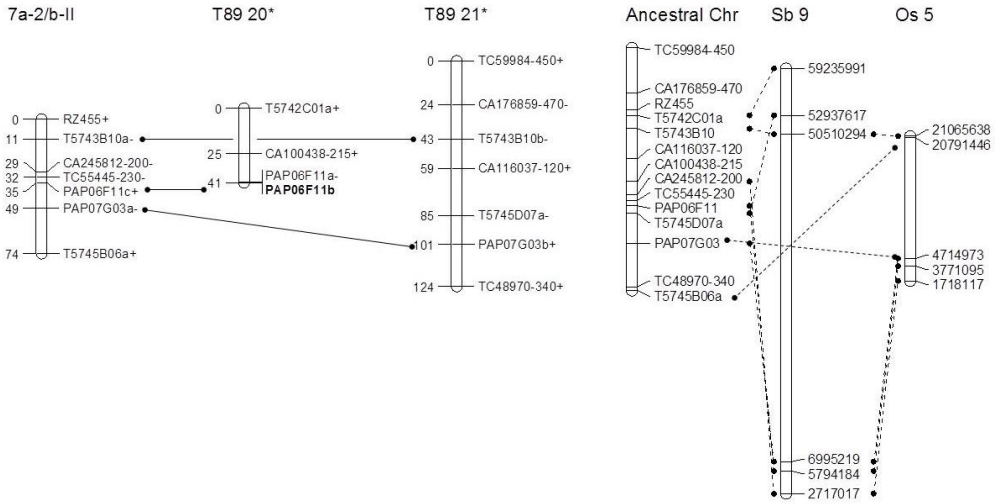
Homologous Group 5 (HG 5)



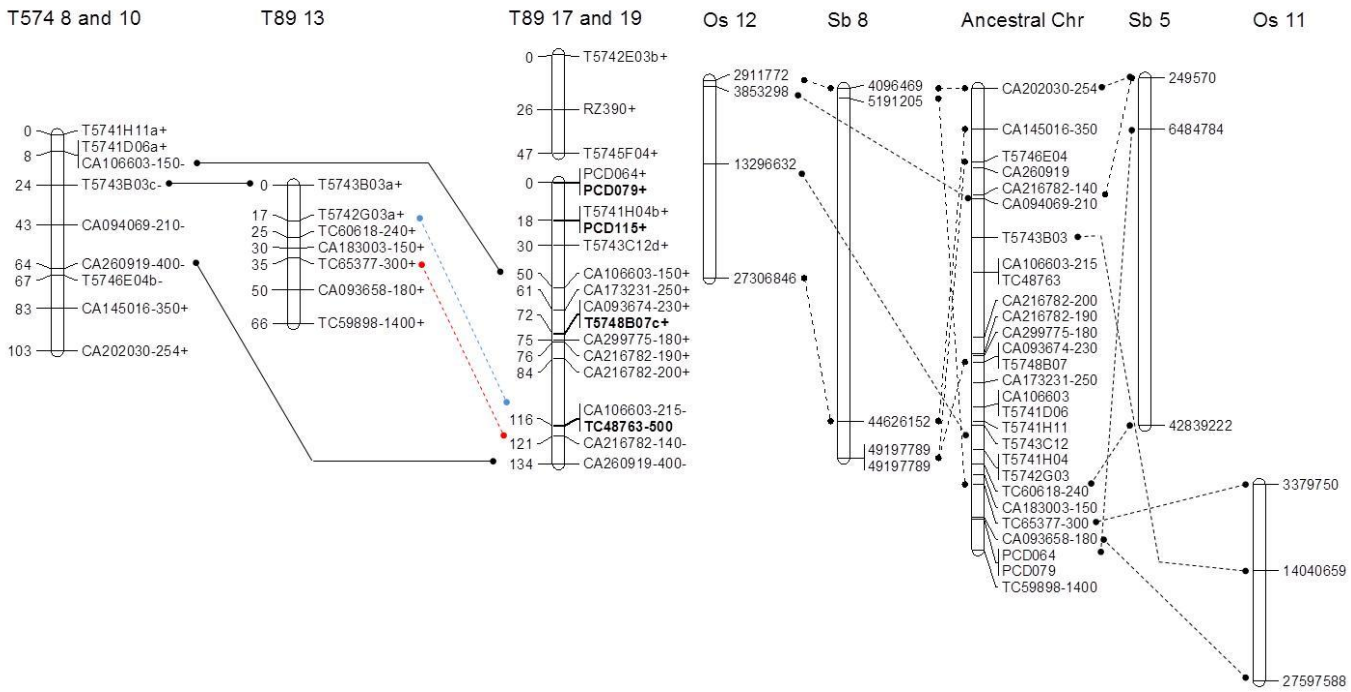
Homologous Group 6 (HG 6)



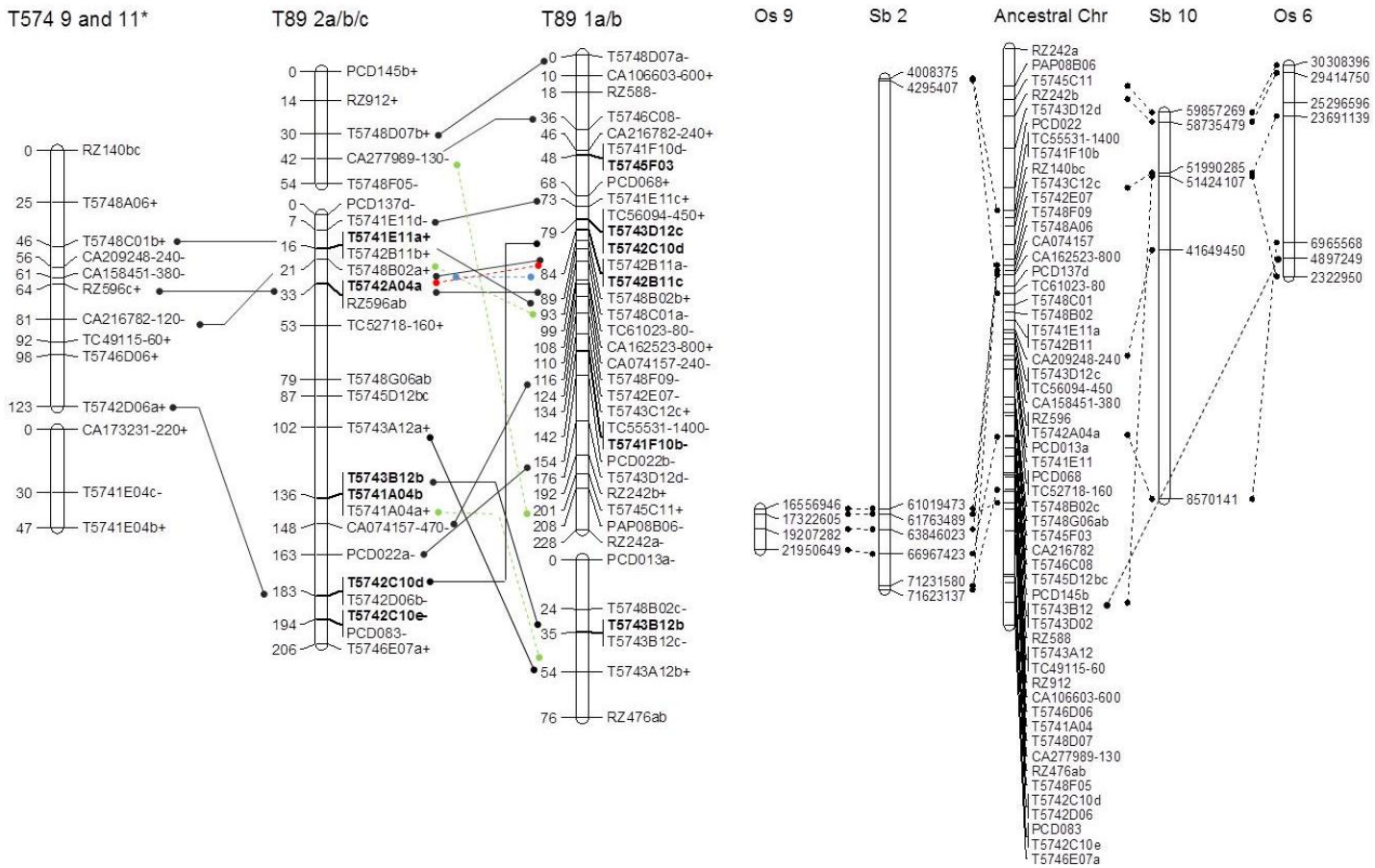
Homologous Group 7 (HG 7)



Homologous Group 8 (HG 8)



Homologous Group 9 (HG 9)

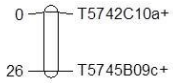


Unassigned CGs

T574 13*

T574 14*

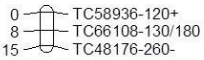
T89 2d



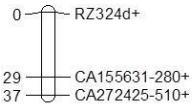
T89 9

T89 10

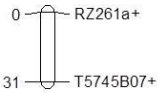
T89 22*



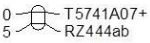
T89 23*



T89 24*



T89 25*



T89 26*

CHAPTER 4

MOLECULAR DISSECTION OF QUANTITATIVE VARIATION IN BERMUDAGRASS HYBRIDS (*CYNODON DACTYLON* X *TRANSVAALENSIS*): MORPHOLOGICAL TRAITS [3]

[3] Khanal, S, Dunne, J.C., Schwartz, B.M., Kim, C., Milla-Lewis, S., Raymer, P.L., Adhikari, J., Auckland, S.A, Rainville, L.K., and Paterson, A.H. 2017. To be submitted to *G3: Genes | Genomes | Genetics*

Abstract

Bermudagrass (*Cynodon* (L.)) is the most important warm-season genus grown for forage or turf. It shows extensive variation in morphological characteristics and growth attributes, but the genetic basis of this variation is little understood. Detection and tagging of quantitative trait loci (QTL) affecting above-ground morphology with diagnostic DNA markers would provide a foundation for genetic and molecular breeding applications in bermudagrass. Here, we report early findings regarding genetic architecture of one foliage (canopy height, HT), two stolon (stolon internode length, ILEN and length of the longest stolon LLS), and two leaf traits (leaf length, LLEN and leaf width, LW) in 110 F_1 individuals derived from a cross between *Cynodon dactylon* (T89) and *C. transvaalensis* (T574). Separate and joint environment analyses were performed on trait data collected across two to five environments (locations, and/or years, or time), finding significant differences ($P < 0.001$) among the hybrid progeny for all traits. Analysis of marker-trait associations detected 74 QTL and 135 epistatic interactions. Composite interval mapping (CIM) and mixed-model CIM (MCIM) identified 32 main effect QTL (M-QTL) and 13 epistatic QTL (E-QTL). Colocalization of QTL for plant morphology partially explained significant correlations among traits. M-QTL qILEN-3-2 (for ILEN), qLLS-7-1 (for LLS), qLEN-1-1 (for LLEN), and qLW-3-2 (for LW) were 'stable' across multiple environments, representing candidates for fine mapping and applied breeding applications. QTL correspondence between bermudagrass and divergent grass lineages suggests opportunities to accelerate progress by predictive breeding of bermudagrass.

Keywords: Quantitative trait locus, QTL correspondence, GMM, QTL Cartographer, QTLNetwork

Introduction

Bermudagrass represents several resilient perennial species of the genus *Cynodon* (L.), which typically colonize tropical, subtropical, and warm temperate regions (Harlan and De Wet 1969). The United States (US) National Plant Germplasm System (NPGS) records eight extant species and ten varieties of the genus, following revised taxonomic classification by Harlan (1970). Common bermudagrass [*Cynodon*

dactylon (L.) Pers.], the most widespread species of the genus, was introduced into the US in the mid-1700's, and has become the most prominent pasture grass of the southern states (Harlan 1970; Wu 2011). It exhibits a wide range of variation for plant characteristics, excellent adaptation and increased biomass in the warmer climates, traffic (wear) tolerance, aggressive growth and recuperative capacity, low input requirements, and drought and salinity tolerance (Beard 1972; Beard and Green 1994; Carrow and Duncan 1998; Hanna et al. 2013; Marcum 2008; Taliaferro 2003). Several triploid hybrids from interspecific crosses between *C. dactylon* (2n = 4x = 36) and *C. transvaalensis* Burtt Davy (2n = 2x = 18; South African bermudagrass) have found tremendous commercial success as lawn or sports turf (e.g., football fields and golf courses) (Florkowski and He 2008; Hanna and Anderson 2008), primarily because they combine stress tolerance of the tetraploid with aesthetic properties of the diploid (Hanna et al. 2013; Taliaferro 2003).

Bermudagrass morphological traits are routinely used to assess phenotypic diversity (Anderson 2005; Si-Yong et al. 2008; Wu et al. 2007), to develop core collections (Anderson 2005; Anderson et al. 2009; Jewell et al. 2012), and to characterize novel germplasm or cultivars (Lu et al. 2009; Taliaferro 2003). Owing to their high heritabilities (Wofford and Baltensperger 1985), morphological traits can be used as selection indices in breeding programs as proxies for correlated, yet much more complex traits (i.e., biomass, abiotic stress tolerance). For example, canopy height, an evaluation of shoot vertical growth, constitutes a significant component of vegetative performance (i.e., biomass, growth rate and habit) (Pittman et al. 2015; Sripathi et al. 2013; Wu et al. 2007) and can find use as an indirect selection index for improved pasture, biomass, and turf characteristics. Similarly, selection of improved wear (or traffic) tolerance can be achieved indirectly by selecting for morphological traits such as internode length (Wood and Law 1974), leaf length (Kowalewski et al. 2015), and leaf width (Kowalewski et al. 2015; Shearman and Beard 1975). Preliminary findings of Kowalewski et al. (2015) suggest that shorter and finer leaves result in increased wear tolerance in bermudagrass. Similarly, stolon growth rate and stolon length were found to be good predictors of establishment speed in *Cynodon* spp. (Magni et al. 2014), *Zoysia* spp. (Patton et al. 2007), and creeping bentgrass (Jones and Christians 2012).

Selection of improved bermudagrass cultivars is based on a number of morpho-phenological traits, growth attributes, and agronomic characteristics (including biotic and abiotic adaptations), and depends upon the intended end-use of the cultivar (for example, as pasture or turf) (Taliaferro 2003). While breeding bermudagrass for forage has primarily relied on traditional practices (i.e., mass selection, recurrent selection), mostly to develop seeded varieties of *C. dactylon* var. *dactylon* (Taliaferro 2003), successful turfgrass breeding programs have utilized both seeded and vegetatively propagated interspecific crosses (between *C. dactylon* and *C. transvaalensis*) as well as natural and induced mutations (Hanna et al. 2013; Wu 2011). Rapid, vertical shoot growth is desirable in pasture for rapid regeneration and increased biomass production, while slow-growing, dwarf or prostrate, drought-resistant varieties are preferred in turf bermudagrass (Mutlu et al. 2015) to reduce mowing and irrigation requirements (Chen et al. 2009; Lu et al. 2008). A number of turf-bermudagrass genotypes, particularly used in golf greens, are spontaneous and irradiated dwarf mutants, particularly those of *C. dactylon x transvaalensis* interspecific hybrids. However, genetic and molecular architecture of dwarf mutation(s) in bermudagrass has not been elucidated (Abernathy 2014). Morphologically, short-statured plants, particularly dwarf mutants of bermudagrass and other grasses, have characteristically shorter leaves and internodes compared to their taller counterparts (Burton et al. 1969; Chen et al. 2016; Lu et al. 2009; Wu et al. 2007). Biologically, plant height is a sum of internode lengths from the base to the uppermost internode plus the length of the spike. Canopy height, on the other hand, encompasses stature-related features including vertical leaf growth, and is therefore a foliage characteristic. Accordingly, significant correlations have been reported among plant height/canopy height, internode length, and leaf length in bermudagrass (Wu et al. 2007) and allied lineages (Cui et al. 2011; Cui et al. 2002; Yu et al. 2014, Sripathi et al. 2013; Zeid et al. 2011). These traits often display continuous phenotypic variation, typical of multigenic control, and exhibit quantitative inheritance, which poses a substantial challenge in bermudagrass breeding. Nevertheless, significant genetic components and moderate to high heritability of these traits permit improvement of both turf and forage bermudagrass by breeding (Wofford and Baltensperger 1985; Wu et al. 2007).

Warm-season turfgrasses lag other crop species in the use of molecular and genomic approaches (Ebina et al. 2014; Hanna et al. 2013). This disconnect is particularly evident in Chloridoids, a clade with ~1,300 species, in which very few genetic linkage maps and QTL analyses have been reported (Bushman and Warnke 2013; Cai et al. 2014; Crawford et al. 2016; Ebina et al. 2014; Guo et al. 2016). Most efforts to date have been invested in zoysiagrass (Cai et al. 2014; Guo et al. 2014), and tef [*Eragrostis tef* (Zucc.) Trotter] (Hewan Demissie and Tatsuhito 2010; Yu et al. 2007; Zeid et al. 2010), and includes a whole genome draft sequence of tef (Cannarozzi et al. 2014) and zoysiagrass (Tanaka et al. 2016). Currently, *Cynodon* spp. lags these other chloridoids in genomic data and molecular breeding applications.

The first genetic linkage framework of bermudagrass (*C. dactylon x transvaalensis*) was based on heterozygosity within each parent (Bethel et al. 2006), mapping the chromosomes of each parent following a pseudo-testcross strategy (Grattapaglia and Sederoff 1994). Recently, we updated this framework with additional simple sequence repeat (SSR) markers found within expressed sequence tags (ESTs) from sugarcane (EST-SSRs) (Khanal et al. 2017a) integrating SSRs with preexisting restriction fragment length polymorphisms (RFLPs) using OneMap (Margarido et al. 2007) (Khanal et al. 2017b). Similar parental linkage maps have been used for QTL detection in pseudo-testcross mapping populations of turfgrasses including creeping bentgrass, *Agrostis* spp. (Honig et al. 2014; Merewitz et al. 2014; Zhang et al. 2012); centipedegrass, *Eremochloa* spp. (Wang et al. 2014), and ryegrass, *Lolium* spp. (Barre et al. 2009; Inoue et al. 2004; Xiong et al. 2006). The updated bermudagrass map is suitable for exploratory QTL analysis since most of the parental genomes (i.e., ~ 80%) lie within 10 cM of a mapped marker (Khanal et al. 2017b), allowing adequate power for QTL detection (Lander and Botstein 1989). We also showed that tetraploid bermudagrass 'is in a relatively advanced state of diploidization' and there appears to have 'substantial subgenome differentiation', 'strong preferential pairing', and 'disomic inheritance', essentially providing a basis to use QTL mapping approaches used in the diploids. Here, we detect and characterize bermudagrass QTL for one foliar (i.e., canopy height), two stolon (i.e., stolon internode length and length of the longest stolon), and two leaf traits (i.e., leaf length and leaf width).

Materials and Methods

Plant Materials and Trial Management

The mapping population consisted of F_1 hybrids from a cross between *C. dactylon* (T89; 4x) and *C. transvaalensis* (T574; 2x) as described (Bethel et al. 2006; Khanal et al. 2017b). For QTL analysis, clonal propagates of a total of 118 F_1s and six standard control varieties (i.e., Tifway, TifSport, Celebration, TifGrand, T-11, and TifTuf) were planted in randomized complete block designs with three replicates at Tifton, GA and Griffin, GA on 9 and 12 June 2010, respectively.

Bermudagrass plugs were used to establish plots ($1m^2$). A granular starter (5N-10P-15K) fertilizer (Agrium U.S. Inc.; Denver, CO) was applied at a rate of 48.8 kg ha^{-1} during establishment and subsequent ammonium nitrate (34N-0P-0K) (Blue Belt Fertilizer Company; Miami, Fl) fertilizer applications were applied monthly at a rate of 24.4 kg ha^{-1} for maintenance. Plots were mowed at 3.61 cm with a rotary mower once a week throughout the project period and clippings were returned to the plot. Irrigation was applied immediately after each fertility application and as needed after mowing to prevent drought stress. ChipCo TopChoice Insecticide (Bayer Environmental Science; Research Triangle Park, NC) was applied once a year for the control of Fire Ants (*Solenopsis* spp.).

Phenotypic Analysis

Seventeen morpho-phenological traits were recorded over a three-year period (i.e., 2010-2012) at Tifton and Griffin. A subset of morphological traits, particularly stolon traits (stolon internode length and length of the longest stolon) and foliage characteristics (canopy height, leaf length, and leaf width), were used in the current study. Canopy heights (HT) were measured (in cm) four times at Tifton (2 and 16 July 2010, 12 July 2011, and 9 July 2012) and thrice at Griffin (2 and 16 July 2010 and 6 July 2012), combining 2010 measurements within locations. Stolon internode length (ILEN) was measured twice in 2011 (20 April at Tifton and 2 June at Griffin) and twice in 2012 (24 September at Tifton; 6 July at Griffin). Measurements from three subsamples of fully elongated internodal distance (in mm) of prostrate stems (third internode)

were averaged across the three replications of each genotype to record ILEN (i.e., nine independent measurements). Length of the longest stolon (LLS; in mm) was recorded twice at Tifton (2 and 15 July 2010) and twice at Griffin (2 and 16 July 2010) during the active growing season. Leaf length (LLEN) was recorded once at both locations in 2012 (11 July at Tifton and 10 July at Griffin). Leaf width was recorded twice at Tifton (13 July 2011 and 11 July 2012) and once at Griffin (10 July 2012). Measurements from three subsamples of fully expanded leaves (in mm) were averaged across three replicates to record LLEN and LW. First measurements of HT and LLS (i.e., 2010) may not represent entire plot as they were scored around a month after planting.

Morphological data was analyzed using the GLM (general linear model) procedure in SAS 9.4 (SAS®, SAS Institute Inc., Cary, NC). The model consisted of the main effects of genotype, location, and/or year or time, and interactions among these main effects. Phenotypic distributions, descriptive statistics, and correlation coefficients between traits were obtained using JMP 12.1.0 (JMP®, SAS Institute Inc., Cary, NC).

Genetic Linkage Maps and QTL Analyses

In the present study, we used EST-SSR markers, SSR-enriched genetic linkage maps, and associated genotypic data from our companion studies (Khanal et al. 2017a; Khanal et al. 2017b), which describes the nomenclature of marker names, cosegregating groups (CGs), linkage group (LGs) and homologous groups (HGs). Biparental and double-dose markers were excluded from interval mapping.

A variety of tools and techniques were explored to decipher candidate QTL underlying complex morphological traits. First, single marker analysis and digenic and trigenic interactions were pursued, the results of which were compared with those from composite interval mapping (CIM) and mixed model-based CIM (MCIM), as follows:

Single marker analysis (SMA)

Eighteen single environment phenotypes (i.e., trait, location, and/or year, or time) pertaining to five morphological traits were independently tested for marker-trait associations against parent-specific markers (T574 or T89). Both simplex markers (i.e., segregating in 1:1 ratio) including biparental markers (i.e., segregating in 3:1 ratio), yielding 'S-QTL'; and duplex markers (i.e., segregating in 5:1 ratio), yielding duplex-QTL ('D-QTL') were used to test the contrast between genotypes (i.e., presence *vs.* absence of an allele) at each marker locus using JMP (at $P = 0.005$). Detailed methodology for SMA is provided in a Supplementary Text File.

QTL interactions in genetic variation

For each single environment phenotype, epistatic associations between two or three loci were extracted using the GMM algorithm implemented in software Genotype Matrix Mapping (GMM) ver. 2.1 (Isobe et al. 2007). We used default search parameters for testing significant associations at combinations of up to three loci and reported marker combinations represented with at least two genotypes. Three independent runs were carried out for each trait (two parent-specific runs and a combined analysis) and all categories of markers (linked or unlinked, simplex or duplex) were assessed for their contribution to the trait. Significant higher order interactions, assessed based on relative F-scores, were favored over the effects of their individual constituents.

Composite interval mapping

The most likely locations of QTL and their genetic effects were detected separately in each parental linkage map by using CIM (Zeng 1994a; Zeng 1994b) employed in software WinQTL Cartographer, version 2.5 (Wang et al. 2007). Single environment phenotypes were used for QTL detection in HT and stolon traits, while combined (across locations and/or years) environment phenotypes were also used for leaf traits. CIM was performed using *Model 6* (Standard Model) with 5 *control markers* as cofactors (as set by *backward regression method*), a *window size* of 10 cM, and threshold value set by permutations (1,000

times) with genome-wide type I error of 5% (Doerge and Churchill 1996). Likelihood ratio (LR) test statistics was calculated at every 2 cM grid position. For closely linked peaks (i.e., within 20 cM), the one with the highest LOD was reported (Tanksley 1993). If QTL (i.e., 2 LOD support intervals) overlapped for a trait in different environments, they were considered coincident and reported as the same QTL (Weinig et al. 2002). Further, QTL detected at lower LOD thresholds (as low as two), if present, were also reported if LOD reaches the permutation-based threshold in at least one of the environments. QTL identifiers included abbreviations of the QTL '*q*' followed by the trait name, its homology group, and ascending numeric values for the number of QTL in the homology group for the same trait.

Mixed-model based CIM

We also performed QTL analysis with a mixed linear model (MLM) approach using MCIM provided in the software QTLNetwork ver. 2.0 (Yang and Zhu 2005; Yang et al. 2007). Single or combined environment phenotypes were used to detect additive effects (α) for main effect QTL (M-QTL) and additive by additive interactions ($\alpha\alpha$) for epistatic QTL (E-QTL). Further, joint analyses of multi-environment phenotypic values were independently run to decipher QTL X environment interactions (QTLx*E*). Significance of QTL detection (i.e., critical F value) was determined with 1,000 permutations at genome-wide type I error of 5%. *Genome Scan Configuration* was set at 10 cM of *Testing Window Size*, 2 cM *Walk Speed*, and 10 cM *Filtration Window Size* with epistatic interactions evoked with *2D Genome Scan*.

Comparative QTL Analyses

Detected QTL were compared with reported studies in other grass species, particularly by targeting QTL identified for the traits of interest (i.e., plant height, stolon internode length, and leaf length and width). Co-localization among QTL were assessed based on a comparative mapping approach employed in our companion study (Khanal et al. 2017b), where we juxtaposed bermudagrass markers against putative orthologous sequences (i.e., sequenced RFLP probes, EST sequences) in rice and sorghum homologs. Thereafter, genomic coordinates of significant marker-trait associations were compared against reported

QTL anchored to physical positions along rice and sorghum genomes, which were accessible through online databases Q-TARO (Yonemaru et al. 2010) and CSGRqtl (Zhang et al. 2013), respectively. Specifically, BLASTn based homology assessment employed in Q-TARO was used to anchor bermudagrass markers to corresponding rice QTL. Similarly, putative orthologous sequences in the sorghum genome identified using significant BLASTn hits (https://blast.ncbi.nlm.nih.gov/Blast.cgi; web accessed: 28 September, 2016) were queried for anchored QTL in CSGRqtl. QTL identifiers followed respective database nomenclatures.

Results

Field Trait Analysis

The distributions of HT, ILEN, and LLS were approximately normal (except HT at Griffin 2010) (Shapiro and Wilk test; $P > 0.05$) and typical of quantitative (i.e., polygenic) inheritance (Figure 4.1), while LLEN at both locations and LW at Tifton were not normal (i.e., not significant at $P < 0.01$). In general, the diploid parent (T574) showed higher trait values for HT, ILEN, LLS, and LLEN, while the tetraploid parent (T89) had characteristically higher mean leaf width at both locations (Table 4.1). Further, trait values for LLS increased (i.e., distribution shifted right), while those for ILEN decreased in the second year. Transgression was not uniform, both in terms of recurrence (i.e., in different environments) and direction (i.e., positive or negative). For example, while most hybrids showed higher LLS values than the parents (i.e., positive transgression) in all four environments, transgression for HT was only evident in 2012. ILEN, LLEN and LW, on the other hand, had different proportions of transgressive segregants in different environments.

The effects of genotype, location, and/or year, or time and their interactions were estimated for five bermudagrass morphological traits based on the general linear models (see "Materials and methods") in combined analysis of variance across environments. Significant differences were found among the clones ($P < 0.001$) for each of the five traits. Further, *G*x*E* interactions were significant for HT and stolon traits but not for leaf traits. Specifically, *genotype* x *location* (environment) interaction was not significant for

the leaf traits i.e., LLEN and LW ($P = 0.258$ and $P = 0.852$, respectively), while all relevant *GxE* interactions (i.e., *genotype* x *location*, *genotype* x *year*/*time* etc.) were significant for HT and stolon traits. As such, QTL analyses using single environment phenotypes (i.e., by location and/or year, or time) were better suited than average values (i.e., across locations and/or years, or times) for HT, ILEN and LLS. On the other hand, insignificant *GxE* estimates for leaf traits provided an opportunity to use means across locations and/or years (i.e., overall values), potentially increasing the power to detect QTL with small effects (Kong et al. 2014). However, we also ran joint analyses for individual traits where location and/or year, or time were included in the QTL models to provide a formal statistical basis for QTL stability instead of recurrence of coincident QTL peaks for single environment phenotypes.

Trait Correlations

Many single environment phenotypes were significantly correlated with each other (Supplemental Table 4.1). All correlations among single environment phenotypes of stolon (ILEN and LLS) and leaf characteristics (LLEN and LW) were moderately significant ($p < 0.001$). However, only seven of ten correlation coefficients among five different environments for HT were significant. Between different traits, most single environment phenotypes of stolon and leaf characteristics were significantly correlated, while a few between HT and the other traits were also significant. However, correlations among LLS and leaf characteristics were not significant. All significant correlations between traits were positive.

Single Marker Analysis (SMA)

Marker-trait associations

Considering single environment phenotypes, a total of 98 associations were significant at $P = 0.005$, of which 28 (28.5%) were also significant at $P = 0.001$ (Supplemental Table 4.2 and 4.3). Based on criterion set for selecting among significant markers within a CG (see "Electronic Supplemental Material"), 19, 53, and two associations at 18 T574, 33 T89, and two biparental marker loci, respectively, were recorded as S-QTL (Supplemental Table 4.3 and 4.4).

Possible associations between 70 duplex markers and the five traits were tested by ANOVA (as for simplex markers). Considering all the traits, 14 significant associations (at $P = 0.005$) at 11 duplex markers were detected, of which eight associated with single environment phenotypes and three with traits in two different environments (Supplemental Tables 4.2 and 4.3). Duplex markers could not be reliably mapped in the genetic linkage maps, but can be superimposed to their closest linkages. Accordingly, four of the 11 duplex markers corresponded to three different T89 CGs. One of the superimposed markers (T5746F07) coincided with an S-QTL at locus T5746H09 mapped to linkage group T89 1c/d. The remaining associations were duplex specific, with the total of 10 duplex markers adding 13 new putative genetic factors (i.e., D-QTL) (Supplemental Table 4.4).

Consistency of S-QTL and D-QTL detection

The consistency of QTL detection for five bermudagrass morphological traits across two (for LLEN), three (for LW), four (for ILEN and LLS), or five (for HT) environments was primarily assessed based on deduced S-QTL and D-QTL detected at a significance threshold of $P = 0.005$. The 74 deduced S-QTL were tagged at 58 marker loci, 45 of which were associated with single environment phenotypes while 10 were associated with two, and one each with three and four trait-environments, respectively. Only six S-QTL pairs tagged at five marker loci were consistent across two different environments for the same trait. Specifically, CA265902-300 was significantly associated with ILEN and LW, CA100438-215 with LLS, and CA162523-100 and CF574110-150 with HT. On the other hand, three pairs of D-QTL were associated with two different environments for the same traits (T5741E11b with HT, T5741E08 with ILEN, and T5746F07 with LLS). All nine consistent QTL (i.e., six S-QTL and three D-QTL) were contributed by the T89 parent.

A priori knowledge of significant marker-trait association in one environment provides a rationale for employing a less stringent threshold (i.e., $P = 0.05$) to identify additional marker-trait associations in different environments. Compared to the total of nine consistent QTL at $P = 0.005$, we found 11, 11, eight,

six, and nine recurring QTL for HT, ILEN, LLS, LLEN, and LW, respectively, which were each detected in at least two environments with $P = 0.005$ in at least one of the environments (Supplemental Table 4.4). Further, two QTL for HT, five for ILEN, two for LLS, and four for LW were consistent across three environments each. Similarly, six LLEN QTL were detected in both environments studied (i.e., Griffin and Tifton).

Epistatic Interactions

Interactions among digenic and trigenic E-QTL

Two (digenic) and three (trigenic) locus combinations were evaluated for epistatic interactions on the measured traits. GMM detected a number of significant combinations (Supplemental Table 4.5, Supplemental Figure 4.1). For two locus combinations, 20 E-QTL were detected for HT, 12 for ILEN, 16 for LLS, 11 for LLEN, and 14 for LW. For three locus combinations, 21 E-QTL were detected for HT, 11 for ILEN, 21 for LLS, six for LLEN, and three for LW. Both parental species contributed equal numbers of E-QTL (57 T574 and 57 T89). Fifteen of the 73 two locus combinations (20%) and six of the 61 three locus combinations (~10%) involved interactions among loci contributed by different parental species. E-QTL were distributed over 13 of 14 T574 CGs and 29 of 34 T89 CGs.

Digenic interactions explained from 16 to 61% of phenotypic variation (i.e., R^2) for HT, 21-78% for ILEN, 16-56% for LLS, 16-44% for LLEN, and 14-47% for LW. R^2 for trigenic interactions were 43-77% for HT, 46-69% for ILEN, 33-79% for LLS, 39-80% for LLEN, and 53-71% for LW. Hence, trigenic interactions showed highest R^2, except that a digenic interaction for ILEN explained more variation (78%) than any three locus combination (69%). Of the total of 135 digenic and trigenic associations, 85 (63%) contained at least one marker with a significant main effect (i.e., S-QTL or D-QTL, $P \leq 0.05$) while 50 (37%) did not involve markers with significant main effects.

Consistency of E-QTL detection

Only one digenic combination involving T574 loci [i.e., T5742A10b(a-) and CA093658-240(a-)] was consistently detected for HT in two different environments (i.e., Griffin and Tifton in 2010). Nevertheless, GMM identified different numbers of common loci among single environment phenotypes for different traits. For HT and LLS, one locus was common across four [RZ574(a-)] and three [RZ717(a-)] different environments, respectively; however, these loci were involved in multiple digenic and trigenic associations [i.e., seven for RZ574(a-), six for RZ717(a-)]. A total of four loci for HT, five for ILEN, 11 for LLS, one for LLEN, and two for LW were involved in a total of 10, 17, 27, three, and five associations, respectively, in two different environments.

Composite Interval Mapping (CIM)

QTL Cartographer identified zero to three QTL for single environment phenotypes for each trait, with totals of seven and 21 QTL contributed by T574 and T89, respectively (Table 4.2, Figure 4.2). Using permutation based thresholds (at 1,000 permutations and genome-wide significance level of 0.05) obtained independently for each single environment phenotype, only three QTL were found to be consistent. Specifically, QTL mapped to T89 16 (qILEN-3-2), T89 20 (qLLS-7-1), and T89 12 (qLW-3-2) for ILEN, LLS, and LW, respectively, were significant in two different environments. With a modified parameter to accommodate thresholds as low as LOD = 2 for recurring trait-specific QTL (see "Materials and Methods"), we identified a total of 28 QTL (41 putative associations delineated by 31 marker intervals), 11 of which were recurrent for the five traits.

Height (HT)

For HT data recorded in five separate environments (i.e. three years at Tifton and two years at Griffin), a total of nine putative QTL (two T574 and seven T89) were identified, explaining 7.5-24% of the phenotypic variation (avg. 14.3%). Putative recurrent QTL for HT included two mapped to T574 and three to T89 (specifically, T574 6a/b and T574 7a-2/b-I, and T89 16, T89 12, and T89 1a/b), all detected in two

different environments. Notably, a number of significant and suggestive QTL peaks in homology group 3 (i.e., HG 3) corresponded to syntenic regions in T574 homologs (T574 3a/b and 12) and T89 homo(eo)logs (T89 3c/d and 16 and T89 12), indicating that the region carries major QTL(s) for HT.

Stolon internode length (ILEN)

Four separate environments for ILEN produced a total of seven putative QTL (four T574 and three T89), which explained 9-23% of phenotypic variation (avg. 17.6%). Recurrent QTL for ILEN included two coincident QTL peaks that were mapped to T89 16 (HG 3) and detected in two different environments. In fact, T89 16 carried QTL at almost overlapping 2 LOD support intervals (i.e., confidence interval) in all four environments, indicating that the linkage group harbors major ILEN QTL. This is further supported by the fact that a suggestive QTL was also detected in T574 at a homologous region (T574 3a/b and 12). The homologous region also corresponds to one explaining significant phenotypic variation for HT. Further, a significant QTL peak in T574 7a-2/b-I and 5a/b and a suggestive peak in T89 8a/b could be associated with the same locus in the two genomes.

Length of longest stolon (LLS)

For LLS data collected twice at Griffin and Tifton during 2012, a total of three putative QTL were detected, all three of which were contributed by T89. These M-QTL explained 9-27% of phenotypic variance. Further, three coincident QTL peaks in T89 20 were detected in three different environments explaining 13-27% of total phenotypic variation for single environment phenotypes, suggesting that it is a site for a major QTL underlying LLS in bermudagrass. Further, a number of suggestive QTL peaks were contributed by T89 homo(eo)logs constituting HG 6. Although significant QTL were not contributed by T574, three suggestive QTL were recurrent in two environments each. Interestingly, these QTL seemingly have suggestive T89 counterparts at homologous regions, indicating that a number of small effect QTL ($R^2 < 10\%$) and a large effect one (T89 20) influence LLS in bermudagrass.

Leaf length (LLEN) and leaf width (LW)

Leaf trait characteristics recorded at Griffin (2012) and Tifton (2011 and 2012) showed a total of 14 putative QTL, five from T574 and the rest from T89. For LLEN, four QTL from T89 explained 10-15% of phenotypic variation and a pair of coincident peaks (constituting QTL qLLEN-1-1) was recurrent in both locations (Griffin and Tifton) as well as in the across-location estimated mean. A total of eight putative QTL (three T574, five T89) for LW explained 10-33.5% of total phenotypic variation. A pair of coincident QTL peaks was mapped to T89 12 and was recurrent in both locations as well as in the across-location means. Multiple overlapping QTL peaks (mapped to T574 7a-2/b-I and 5a/b) represented a T574 QTL for LW, the most prominent one of which explained 33.5% of variation for the trait.

Mixed-model Based CIM

Additive effects and additive x E interactions

Separate analysis of single environment phenotypes detected a total of seven unique M-QTL, four for ILEN, one for LLS, and two for LW (Table 4.3). No QTL were detected for HT and LLEN based on input parameters for QTL detection (see "Materials and Methods"). Five of the seven M-QTL were contributed by T89, and two by T574. Five of the seven QTL were also detected by CIM and were identified with CIM QTL identifiers, while two novel QTL specific to ICIM were assigned new identifiers (i.e., qILEN-1-1 and qILEN-3-4). Most additive QTL conditioned an increase in trait values (suggesting that the dominant alleles decreased trait values) except that qILEN-1-1 decreased internode length.

Joint analysis of each trait to decipher genotype x environment (*G*x*E*) interactions detected two additive x environment effects (*αe*, Table 4.3). Specifically, qILEN-3-2 showed significant *αe* effect for ILEN data collected at Tifton in 2012 (*αe*4), while qLLS-7-1 showed significant interactions with LLS data collected at Griffin in 2011 (*αe*1) and Tifton in 2012 (*αe*4). The two QTL were consistently detected across two to three environments using SMA, CIM, and MCIM following single environment QTL analysis.

Epistatic effects and epistasis x E interactions

Three additive x additive QTL (*αα*, Table 4.4) were detected for single environment phenotypes, two of which involved T89 loci. Interestingly, two of the three epistatic pairs carried M-QTL identified by CIM. Specifically, qILEN-6-1/qILEN-5-1 and qLW-3-1/qLW-3-2 showed significant *αα* interactions in G12 (i.e., Griffin 2012) and all four were also detected by CIM as bearing M-QTL effect. A third *αα* interaction (i.e., qLLS-5-1/qLLS-2-1) also carried a suggestive QTL qLLS-5-1 (i.e., LOD=3). As such, all three *aa* interactions involved putative M-QTL.

In the joint analysis, a total of 22 loci were involved in epistatic interaction in 11 digenic combinations, five of which had at least one M-QTL (Table 4.4). For all new loci involved in epistatic interactions, new QTL identifiers were provided. Concerning estimated effects of the digenic interactions, one pair had negative and eleven pairs had positive effects, while two of the 11 interactions also displayed *αα* x *ααe* effects.

Genomic Hubs Carrying Colocalized QTL

In SMA analysis, eight markers mapping to six different LGs and three unlinked markers were involved in 29 associations with at least two different traits (Supplemental Table 4.4). One of the eight markers (RZ140bc) originated from T574 while the rest affiliated with T89. Interestingly, putative S-QTL effects were in the same direction in all cases. For example, three shared markers (CA162523-100, RZ900, and T5741A10a) conditioned a decrease, while one (PCD053c) increased trait values for HT and LLEN simultaneously. Further, one common digenic interaction involving T574 loci [T5745B06a(--) and T5741H06a(a-)] was detected for HT and ILEN in Tifton 2012, which conditioned a decrease in both trait values following GMM analysis. Similarly, two major genomic hubs carried multiple QTL for bermudagrass morphological traits based on CIM. HG 3 and associated LGs from both parental genomes (T574 3a/b and 12, T89 3c/d and 16, and T89 12) carried several QTL peaks associated with HT, ILEN, LLEN and LW. Similarly, T89 20 (constituting HG 7) carried recurrent QTL peaks for LLS (detected in

three different environments) and two other significant QTL, one each for HT and ILEN. Significant positive correlations among five morphological traits were partially explained by the presence of colocalized QTL showing effects in the same direction. However, the genetic basis of relationships between the traits and high coincidence among QTL underlying bermudagrass morphology may be due to either pleiotropy or tight linkages and needs further assessment.

QTL Correspondence

Homology based search for anchored QTL in rice and sorghum genomes showed that at least 33 significant marker-trait associations (QTL) (corresponding to 29 markers) identified in our study colocalize with target QTL in other grasses at putative orthologous and/or syntenic regions (Supplemental Table 4.2). For example, a number of significant marker-trait associations were detected for HT at putative syntenic regions in bermudagrass LGs constituting HG 03 [i.e., T574 3a/b and 12 and T89 3cd and 16], which represented one of the two genomic hubs carrying colocalized QTL. Specifically, a cross-transferable *Pennisetum* marker (i.e., PAP02D11) mapped to T89 12 was significantly associated with HT at Tifton (2011). The orthologs of PAP02D11 were present in syntenic chromosomal regions of rice (*Os 1*) and sorghum (*Sb 3*) and anchored a number of plant height QTL in both, including a semi-dwarfing gene (i.e., *sd-1*) in rice. Plant height QTL in sorghum (i.e., Q.Lin1995.HtAvg-1-1) and sugarcane (i.e., Q.Ming2002a.PLHT.11_3-1) were also associated with a cross-transferable sugarcane marker (i.e., CF574110) that mapped adjacent (~10 cM) to PAP02D11 and showed significant associations with canopy height in two different environments. Another *Pennisetum* marker (i.e., PAP07C04a) which mapped to the homo(eo)logous T574 LG (i.e., T574 3a/b and 12) was also anchored to the two plant height QTL tagged at CF574110 loci and showed significant association with canopy height in one of the environments (i.e., Tifton 2010). Similarly, a second genomic hub with colocalized QTL (i.e., HG 07) carried markers orthologous to a putative syntenic region of a sorghum chromosome (i.e., *Sb 9*) anchoring sorghum plant height QTL (i.e., Q.Lin1995.HtM-9-1 and Q.Brown2006.PTHT-9-1).

Discussion

In the present study, we enhance molecular and genomic information for one of the most important warm-season turf genera, *Cynodon*, reporting QTL analyses using SSR-enriched genetic linkage maps and morphological trait data collected across two different locations and/or two to three different years, or times (i.e., two to five environments). To date, only QTL analysis using biparental segregants was reported for establishment rate in common bermudagrass population (Guo et al. 2016). Based on an F_1 population resulting from interspecific hybridization between common bermudagrass and African bermudagrass that have contrasting phenotypic and agronomic characteristics and condition heterotic attributes to their progenies, QTL identified in this study are directly relevant to major bermudagrass turf cultivars and are candidates for improvement in similar pedigrees.

A genetic map with DNA markers within 10 cM of most of the genome (~80% coverage) allowed us to use SMA and GMM together with standard interval mapping approaches (i.e., CIM and MCIM) to dissect complex morphological traits. Following SMA, we detected a total of 112 significant marker-trait associations (at $P = 0.005$ with estimated 8-20% false positives), 33 of which showed correspondence with anchored QTL in orthologous/syntenic regions of allied genomes. Based on corresponding associations in different environments at a lower threshold ($P = 0.05$) we deduced a total of 45 recurrent associations for the five traits, a subset of which were also detected by CIM and MCIM procedures and have promise for applied breeding applications. While most QTL conditioned a decrease in trait values, a third (i.e., 27/81 QTL detected by SIM and 14/40 QTL detected by CIM or ICIM) were associated with increases, suggesting that there is scope for divergent selection in bermudagrass. The current study was based on QTL analyses in separate parental linkage maps. T89 contributed more QTL, consistent with its tetraploidy and more markers mapped to it, but each parent contributed to QTL effects in both directions for each trait under consideration. However, we could not make unequivocal assertions about QTL correspondence between T574 and T89, particularly because of a paucity of allelic homologs (i.e., common markers) between the

two maps, which curtailed our ability to evaluate gene dosage effects. As such, there is a need for high density integrated linkage maps built with codominant markers and biparental bridges (i.e., markers segregating in 3:1 ratio), as elaborated in our companion paper (Khanal et al. 2017b).

Genetic Basis of Bermudagrass Morphological Traits

Canopy height in turfgrasses is a function of both stem and leaf extension, a fact that was evident from several significant correlations between HT, stolon (particularly ILEN), and leaf traits (particularly LLEN), further supported by a number of coincident QTL among traits (more so with ILEN and LLEN). Significant positive correlation between plant height and leaf length was also reported in rice (Cui et al. 2002; Yan et al. 1999), switchgrass (Sripathi et al. 2013), and perennial ryegrass (Yamada et al. 2004), and co-localized QTL were also evident in some studies (Cai et al. 2015; Yan et al. 1999), suggesting that the genetic basis of plant height and leaf length are partially overlapping. In the current study, four markers were significantly linked with both traits - two of which were also detected by CIM. Similarly, five markers were significantly associated with HT and ILEN, while CIM detected two coincident QTL at the two genomic hubs – the result is consistent with prior studies reporting significant positive correlations partially explained by the presence of colocalized QTL [for example: tef (Yu et al. 2007; Zeid et al. 2011); wheat (Cui et al. 2011; Yu et al. 2014)]. For HT, SMA produced 17 deduced QTL (including three D-QTL; eight recurrent) and CIM/ICIM detected 11 QTL (average R^2=13.6%) including five recurrent (i.e., in at least two different environments), one E-QTL ($R^2_{\alpha\alpha}$=1.1% in joint analysis), and a significant '*Epistasis*x*E*' interaction ($R^2_{\alpha\alpha e}$=1.9%), which partially explains weak correlations, significant *GxE*, and indicates discrete and multigenic regulation of the trait in different environments - an observation reiterated in other grasses (Pauly et al. 2012; Poncet et al. 2000).

Significant correlations among single environment phenotypes of stolon characteristics indicated that we may be able to find recurrent QTL in different environments. Accordingly, a total of 24 (with 11 recurrent; three D-QTL) and eight (all recurrent including 1 D-QTL) deduced QTL were identified for

ILEN and LLS. CIM/ICIM detected 13 QTL (average R^2=13.4%) for ILEN that included two recurrent and four E-QTL ($R^2_{\alpha\alpha}$=14% in joint analysis), while seven QTL (average R^2=13.5%) were detected for LLS that included two recurrent and four E-QTL (average $R^2_{\alpha\alpha}$=5.3% in joint analysis). Based on QTL recurrence, stolon traits were seemingly more 'stable' than canopy height.

Wofford and Baltensperger (1985) reported moderately high heritabilities associated with LLEN and LW in common bermudagrass. In the current study, *GxE* (i.e., location) interaction for leaf traits was not significant, which is consistent with Wofford and Baltensperger (1985) and Kowalewski et al. (2015), and provided a statistical basis for combined data analysis across years for the two traits. We thus used separate environment phenotypes as well as combined analysis for QTL detection. Phenotypic distributions of the leaf traits (specifically, LLEN at both locations and LW at one) approached but were not exactly Gaussian (i.e., normality rejected by Shapiro-Wilk test), suggesting an oligogenic mode of inheritance. However, leaf length is often characterized as a trait governed by many genes with small effects and profoundly influenced by growth environment (Barre et al. 2009; Barre 2015; Marousky et al. 1992). In the current study, a total of 13 deduced QTL from SMA (including six recurrent QTL) and eight CIM/ICIM-detected QTL (average R^2=12.3%) including one recurrent (repeatedly detected in separate and joint environment CIM analyses) and two E-QTL ($R^2_{\alpha\alpha}$=3% in joint analysis with MCIM) were identified for LLEN.

For LW, seventeen SMA-based QTL (including seven recurrent; four D-QTL) and nine CIM/ICIM-detected QTL (average R^2=16.5%) including two recurrent QTL and two E-QTL (average $R^2_{\alpha\alpha}$=20.6%) were identified in the current study. In a comparable study in *Agrostis* (Zhang et al. 2012), three leaf width QTL collectively explained 23% of phenotypic variation. QTL effects were higher in our study, with multiple regression estimates involving two to four SMA-derived QTL explaining 27.2-69.4%, while two to three CIM-detected QTL collectively explaining 30.1-48.2%. of phenotypic variation in three different environments. A genomic region in LG 7a-2/b-I carried tandem QTL (i.e., qLW-5-1 and qLW-5-2)

contributed by T574 (the parent with finer leaves) that conditioned a decrease in leaf width and explained 13.3-33.5% of phenotypic variance in Tifton in separate and joint environment analyses (i.e., across years). In rice, Liu et al. (2015) reported a presence of closely linked leaf width QTL in chromosome 3 that were stable across 5 different environments. We note that our tandem LW QTL do not correspond with the ones reported by Liu et al. (2015), but detection of closely linked QTL contributing significantly to the same trait in diverse lineages is noteworthy.

While a majority of QTL conditioned a decrease in LLEN and LW (i.e., heterozygotes with decreased trait values), regardless of their parental origin, a few also increased trait values. The hybrid bermudagrass population showed significant positive correlations for the leaf traits, despite contrasting parental characteristics, and yet common putative QTL were not detected. Significant correlations between these leaf traits have also been reported in other grass lineages (Wang et al. 2014; Yamada et al. 2004). However, QTL colocalization between leaf traits is often lacking or limited, as observed in the current study. QTL correspondence was completely lacking in perennial ryegrass (Yamada et al. 2004), centipedegrass (Wang et al. 2014), sorghum (Lu et al. 2011), and Brachiaria (Thaikua et al. 2016), while few colocalized QTL were reported in rice (Bing et al. 2006; Cai et al. 2015; Liu et al. 2015; Wang et al. 2012; Yan et al. 1999; Zhang et al. 2015) and wheat (Wu et al. 2016). In a maize nested association mapping (NAM) population, leaf length and width displayed weak correlations and largely discrete putative causative variants underlying the traits (Tian et al. 2011). Apparently, leaf length and width share few pleiotropic or closely linked genetic determinants.

QTL Correspondence Among Grasses

Although many turf-bermudagrasses (particularly those used in the golf greens) are dwarf mutants, genetic architecture of dwarf mutation(s) in bermudagrass has not been elucidated (Abernathy 2014); unlike several grass lineages where molecular and physiological basis of dwarfing are well characterized. For example, rice chromosome 1 (*Os* 1) carries a number of plant height QTL (Thomson et al. 2003) apparently

coincident with dwarfing genes *d-10* (Huang et al. 1996; Yu et al. 1991) and *d-18* (Huang et al. 1996; Ideta et al. 1993; Xiao et al. 1992) and a semi-dwarfing gene *sd-1* (Cho et al. 1994; Huang et al. 1996; Li et al. 2003). The 'green revolution gene', *sd-1* encodes a mutant enzyme involved in gibberellin synthesis and imparts short-stature (i.e., semi-dwarfing) to rice (Monna et al. 2002; Spielmeyer et al. 2002). A QTL (i.e., *ph1*) close to the *sd-1* locus, has been repeatedly detected in different QTL studies and two genes of interest (i.e., *OsGA3ox2* gibberellin biosynthetic gene and *D2* brassinosteroid biosynthetic gene) have been identified and isolated from the vicinity of *ph1* (You et al. 2006). Putative orthologous regions around *sd-1* have also been reported to carry plant height QTL in maize (Austin and Lee 1996; Khairallah et al. 1998; Schön et al. 1994), *Setaria* (Mauro-Herrera and Doust 2016), sorghum (Zhang et al. 2015), and switchgrass (Mauro-Herrera and Doust 2016; Serba et al. 2015). Notably, bermudagrass HG 3 also seems to carry gene(s) conditioning plant morphology, particularly relevant to plant stature, at regions homologous to the semi-dwarfing *sd-1* locus. Accordingly, a number of non-random correspondences of QTL locations between bermudagrass and allied grasses were detected. For example, being detected twice at Tifton (TT1 and TT2) and attaining marginal significance (LOD = 3.3) at GT1 following CIM and MCIM, qLLS-7-1 was one of the 'stable' QTL that conditioned a decrease in LLS and explained significant proportions of phenotypic variation (i.e., 13.7% to 23.1%) of the trait in different environments. A canopy height QTL (i.e., qHT-7-1) was also coincident with qLLS-7-1. Markers flanking the QTL region (i.e., CA100438 and PAP06F11a) had putative orthologs at the syntenic region of a homologous sorghum chromosome (i.e., *Sb 9*) that anchored two plant height QTLs (i.e., Q.Lin1995.HtM-9-1, Q.Brown2006.PTHT-9-1). Our results reiterate the assertion that genes/QTL for 'domestication' traits often correspond across divergent lineages (Paterson et al. 1995) providing a leverage to accelerate genetic gains in predictive breeding of marginalized grasses like bermudagrass.

Implications for Bermudagrass Breeding

We found significant genotypic effects ($P < 0.001$) and positive correlations among the morphological traits, consistent with a prior study in bermudagrass (Wu et al. 2007). Exploratory analyses produced four key observations of interest in bermudagrass breeding: first, epistatic interactions were important and mostly involved M-QTL; second, QTLx*E* interactions were scarce and of comparatively lower magnitudes; third, a number of QTL underlying the traits of interest were colocalized; and fourth, both parents contributed QTL with contrasting phenotypic effects.

Epistatic interactions are important components of trait variation including plant morphology in grasses (Guo and Hong 2010; Ku et al. 2012; Wang et al. 2012; Yang et al. 2016). In the current study, significant epistatic interactions ($R^2\alpha\alpha$ = 1.1-22.5%) were detected by MCIM for each of the five traits in separate and/or joint environment analyses. Most epistatic interactions (64%) identified by GMM involved at least one significant S-QTL. In fact, a number of S-QTL (30) detected at a stringent significance threshold (avg. P = 0.001) with high main effect contribution to the traits (avg. R^2 = 13%) were also involved in epistatic interactions. Further, 9 of 16 $\alpha\alpha$ interactions identified by MCIM had at least one M-QTL. Interestingly, two of the E-QTL involved digenic interactions between previously detected pairs of M-QTL. For example, CIM detected significant main effects for QTL qILEN-6-1 and qILEN-5-1 for stolon internode length and qLW-3-1 and qLW-3-2 for leaf width, while their interactions were also significant in both separate and joint environment analysis following MCIM. On the other hand, QTLx*E* interactions were largely absent - a few inconsistent cases contributed relatively scantly ($R^2\alpha e$ = 0.7-1.2%; $R^2\alpha\alpha e$ ~2%) to overall trait variances (Table 4.3), indicating that detected QTL are mostly 'stable'. Besides, colocalization of QTL (Table 4.2, Supplemental Table 4.4) suggests that the genetic base of stolon and foliage morphology (except that between LLEN and LW, and between LLS and leaf traits) could be determined by pleiotropic or tightly linked genes, indicating the feasibility of simultaneous improvement of the target traits by breeding. QTL correspondence with allied grasses showed that several

orthologous/syntenic regions harbor plant morphology QTL, not only those of interest in the current study (Supplemental Table 4.2), but also involving seedhead-related characteristics (data not shown). However, pleiotropy *vs.* tight linkage and conservation of orthologous function in divergent lineages are issues that may be clarified by fine mapping and characterization of causative allelic variants at these loci. Further, the parental species of the current study contributed alleles that condition contrasting phenotypic effects in the hybrid population, which indicates that both carry favorable alleles suited for alternative breeding goals (i.e., turf or forage breeding).

Phenotypic recurrent selection has been successful in conditioning turf morphological traits in *C. dactylon* var. *dactylon* breeding for seeded bermudagrass cultivars (Taliaferro 2003). QTL analyses suggest that molecular breeding approaches like marker-assisted or genomic selection are feasible. It is in this context that we would like to reiterate that our mapping population is an F_1 interspecific triploid, a reproductively sterile population that cannot be subjected to population breeding approaches. Nevertheless, selection of parents or their hybrids, hitherto exclusively dependent upon phenotypic selection, can be aided by marker-assisted selection (MAS). In intraspecific *C. transvaalensis* populations studied in greenhouse environments, genetic variance of plant height, internode length, stolon length, and leaf length constituted both additive and dominance components (the latter contributing more to the total), suggesting that clonal selection of F_1 hybrids is desirable for improving the traits under consideration (Kenworthy et al. 2006). Owing to the limitation of pseudo-testcross mapping, we could not partition QTL effects into additive or dominance components in the present study. The QTL identified in this study, especially qILEN-3-2 (for ILEN), qLLS-7-1 (for LLS), qLEN-1-1 (for LLEN), and qLW-3-2 (for LW) represent potential candidates for fine mapping and for further validation in bermudagrass breeding populations. QTL correspondence between bermudagrass and other grasses suggests that ‘predictive breeding’ may be a fruitful avenue for improving traits of interest in bermudagrass.

Conclusion

In the present study, we dissected the genetic basis of complex morphological traits of interest in turf bermudagrass. A number of genomic regions with significant contributions to the traits were detected, which can be targeted in fine mapping and introgression breeding. This early QTL study in bermudagrass adds to molecular breeding resources and information in the genus. There is much potential for advancing current research by increasing the size of the mapping population, increasing marker densities using state-of-the-art genomic tools, integrating parental linkage maps, and collecting more extensive phenotype data (i.e., multi-environment across several locations over multiple years). Turfgrass performance (i.e., establishment and recovery speed, canopy density and uniformity after mowing, and other aesthetic properties) also varies with different management strategies (i.e., mowing height, mowing frequency, fertilizer input, growth-regulator application) and different environmental conditions. Therefore, QTL studies under different management plans and/or growth environments are also warranted.

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Table 4.1 A summary statistics of morphological traits in F_1 mapping population and parental lines used for QTL mapping in *C. dactylon* x *transvaalensis*.

Trait[a]	Env.[b]	Parental means		F_1 population	
		T89	T574	Mean (s.d.)	Range
HT	G10	1.83	8.99	3.74 (1.3)	1.83-10.33
	T10	3.99	14.16	7.35 (1.9)	3.67-13.00
	T11	9.80	15.10	17.73 (4.9)	8.22-33.11
	G12	7.60	12.33	11.78 (2.0)	6.67-16.00
	T12	11.00	11.66	12.58 (3.1)	5.33-20.33
ILEN	G11	16.22	27.77	25.07 (3.9)	14.89-36.22
	T11	17.66	22.77	20.63 (3.7)	10.89-30.56
	G12	17.54	21.53	21.94 (3.5)	13.44-31.47
	T12	12.18	10.72	15.34 (2.9)	8.41-23.24
LLS	G10-T1	17.66	22.66	35.42 (6.3)	19.67-49.67
	T10-T1	28.00	31.66	37.80 (6.1)	22.00-50.00
	G10-T2	31.66	26.66	51.11 (9.3)	29.67-74.67
	T10-T2	43.00	45.00	64.13 (10.6)	41.00-90.33
LLEN	G12	22.74	34.35	31.31 (4.9)	20.96-45.88
	T12	21.13	25.02	24.53 (5.2)	14.19-44.07
LW	T11	2.16	1.61	1.98 (0.2)	1.54-2.59
	G12	2.26	1.64	1.72 (0.2)	1.34-2.19
	T12	2.06	1.66	1.79 (0.2)	1.44-2.39

[a] *HT* Canopy height (in cm, five environments), *ILEN* internode length (in mm, four environments), *LLS* length of the longest stolon (in mm, four environments), *LLEN* leaf length (in mm, two environments), and *LW* leaf width (in mm, three environments).
[b] Environment identified with prefixes *G* Griffin and *T* Tifton followed by *10* (2010), *11* (2011), *12* (2012) and T1 (1st date) or T2 (2nd date).

Table 4.2 Composite interval mapping of bermudagrass morphological traits in *C. dactylon* (T89) x *transvaalensis* (T574) F_1 population.

QTL	Source	Linkage group[a]	Env.[b]	Nearest marker	Position	Marker interval[c]	LOD	Effect	R^2 (%)
Height (HT)									
qHT-1-1	T89	4c/d-I and 18	T10	T5741C03c	146.4	T5748G02b - CA181199-230	3.9	-1.65	15.4
qHT-2-1	T89	3a/b	G12	TC52187-250	79.0	T5743E12a - T5745A03ab	3.4	-1.42	12.6
qHT-3-1	T89	16	T10	CA265902-300	59.9	RZ543a - CA186643-90	3.0†[d]	-1.22	9.2
			T12	CA186643-90	90.8	T5746E10b - T5741D01ab	4.3	+2.49	15.2
qHT-3-2	T89	12	T11	PAP02D11	0.0	PAP02D11 - CF574110-150	3.9	-3.71	12.7
			G12	CF574110-150	10.0	PAP02D11 - TC48402-320	2.2†	+1.09	7.5
qHT-5-1	T574	7a-2/b-I and 5a/b	T10	RZ401ab	52.6	CA176859-320 - CA106832-90	3.6	+1.51	14.9
			G12	RZ401ab	50.6	CA176859-320 - TC69998-500	2.6†	+1.68	15.9
qHT-6-1	T574	6a/b	G10	T5743C03d	69.2	TC66673-70 - T5741C09ab	2.2†	-0.80	9.6
			T10	T5743C03d	61.4	TC66673-70 - T5741C09ab	3.1	-1.89	24.1
qHT-7-1	T89	20	T10	CA100438-215	24.7	T5742C01a - PAP06F11a	4.7	-1.53	15.6
qHT-9-1	T89	1a/b	G10	TC55531-1400	142.2	T5743C12c - PCD022b	2.3†	-0.72	7.5
			G12	T5742E07	127.8	T5748F09 - T5743C12c	3.6	+1.86	21.4
qHT-UN-1	T89	24	T11	T5745B07	18.1	RZ261a - T5745B07	3.6	+4.54	18.1
Internode length (ILEN)									
qILEN-3-1	T89	16	G11	T5743A03	0.0	T5743A03 - RZ543a	2.4†	+2.40	9.4
			G12	RZ288	24.1	T5743A03 - RZ543a	3.5	+3.16	19.5
qILEN-3-2	T89	16	G11	CA265902-300	59.1	RZ543a - CA186643-90	4.0	-2.64	11.3
			T11	CA265902-300	63.9	CA162523-100 - CA186643-90	5.1	-3.33	19.6
qILEN-3-3	T574	3a/b and 12	T11	T5746F06	84.4	TC61023-180 - T5743D06b	6.2	-3.52	21.6
qILEN-5-1	T574	7a-2/b-I and 5a/b	G12	T5741A08bc	8.0	T5741A08bc - T5745F10b	3.6	+3.46	23.3
qILEN-6-1	T574	6a/b	G12	CA182108-410	0.0	CA182108-410 - T5745B01	3.8	-2.62	13.2
qILEN-7-1	T89	20	G11	CA100438-215	30.7	T5742C01a - PAP06F11a	3.4	-3.70	22.4
qILEN-9-1	T574	9 and 11	T11	RZ140b	12.0	RZ140bc - T5748A06	3.8	-3.22	18.5
Length of the longest stolon (LLS)									
qLLS-7-1	T89	20	GT1	CA100438-215	34.7	CA100438-215 - PAP06F11a	3.3†	-6.07	22.9
			TT1	CA100438-215	24.7	T5742C01a - PAP06F11a	4.5	-4.49	13.4
			TT2	CA100438-215	26.7	T5742C01a - PAP06F11a	7.1	-11.24	27.2
qLLS-9-1	T89	2a/b/c	GT1	PCD145b	0.0	PCD145b - RZ912	4.0	-5.21	14.2
qLLS-9-2	T89	1a/b	TT1	PCD068	60.2	T5741F10d - T5741E11c	4.5	+6.05	24.4
			GT2	T5741F10d	48.2	T5746C08 - PCD068	2.6†	+5.85	9.1
Leaf length (LLEN)									
qLLEN-1-1	T89	4c/d-I and 18	G/T12	T5742F09c	96.2	T5741E07c - CA156680-150	3.8	-3.10	11.5
			G12	T5742F09c	93.3	T5741E07c - CA156680-150	2.7†	-3.25	10.6
			T12	T5742F09c	96.2	T5741E07c - CA156680-150	4.0	-3.75	11.7
qLLEN-3-1	T89	16	T12	T5741D01ab	96.8	T5746E10b - T5742A10a	3.4	+3.35	9.8
qLLEN-3-2	T574	3a/b and 12	G/T12	TC56094-130	28.4	CA219295-140 - T5745H10de	4.0	-3.72	14.7
qLLEN-3-3	T574	3a/b and 12	G/T12	TC61023-180	66.3	CA272425-230 - PCD131f	3.9	-4.58	23.8
qLLEN-4-1	T89	T89 14 and 27	G/T12	TC67261-150/230	0.0	TC67261-150/230 - T5745B05	3.5	-2.93	10.6
qLLEN-9-1	T89	1a/b	G12	T5742E07	122.2	T5748F09 - T5743C12c	3.9	+3.89	14.8
Leaf width (LW)									
qLW-1-1	T89	4c/d-I and 18	G12	T5741C03c	148.4	T5748G02b - CA181199-230	3.6	+0.15	14.9
qLW-2-1	T89	3a/b	T12	TC62694-450	95.8	T5745A03ab - RZ717	3.4	-0.11	10.0
qLW-3-1	T89	16	G/T12	CA265902-300	59.1	CA162523-100 - CA265902-300	4.1	-0.11	13.6
			G12	RZ543a	36.1	RZ288 - CA162523-100	4.5	+0.18	21.3
qLW-3-2	T89	12	G/T12	RZ543b	31.1	CF574110-150 - T5746E10a	3.4	-0.10	11.9
			G12	TC48402-320	27.1	CF574110-150 - RZ543b	3.6	-0.13	12.0
			T12	RZ543b	34.5	CF574110-150 - T5746E10a	3.4	-0.11	10.3
qLW-3-3	T574	3a/b	G/T12	T5742A10b	26.3	T5741B04 - RZ995	3.3	+0.14	23.3
qLW-5-1	T574	7a-2/b-I and 5a/b	T11/12	PCD148ab	141.4	PCD148ab - T5748F06b	3.4	-0.18	33.5
			T12	PCD148ab	143.4	CA078499-430/600 - T5748F06b	2.2†	-0.16	20.9
			T11	PCD148ab	135.4	PCD148ab - T5748F06b	2.8†	-0.14	13.3
qLW-5-2	T574	7a-2/b-I and 5a/b	T11/12	CA078499-430/600	125.5	CA148529-220-PCD148ab	4.1	-0.12	13.9
			T11	CA078499-430/600	125.5	CA148529-220-PCD148ab	5.3	-0.17	16.8
qLW-7-1	T89	21	G/T12	T5743B10b	37.9	CA176859-470 - T5743B10b	4.3	-0.14	22.7

[a] Linkage group nomenclature following our companion paper. [b] Environment identified with prefixes *G* Griffin and *T* Tifton followed by *10* (2010), *11* (2011), *12* (2012) and T1 (1st date) or T2 (2nd date). [c] Markers spanning 2-LOD support interval. [d] QTLs that do not meet permutation threshold and are nominally significant (LOD = 2).

Table 4.3 Additive (α) and additive x environmental (αe) effects underlying bermudagrass morphology detected by mixed-model based CIM (MCIM) procedure implemented in QTL Network v. 2.0.

Trait	QTL	Env.[a]	Source	Interval	Linkage group[b]	α[c]	$\alpha e1$[d]	$\alpha e2$[e]	$\alpha e3$[f]	$\alpha e4$[g]	R^2_{α} (%)[h]	$R^2_{\alpha e}$ (%)[i]
ILEN	qILEN-3-2	Joint	T89	CA265902-300 - T5746E10b	16	-2.02****	NS	NS	NS	+0.79*	5.1	0.7
	qILEN-1-1†[j]	G11	T89	T5743D12e - PCD021b	4c/d-1 and 18	+2.63****					25.6	
	qILEN-3-2	G11	T89	CA265902-300 - T5746E10b	16	-3.10****						
	qILEN-3-2	T11	T89	CA265902-300 - T5746E10b	16	-3.65****					17.6	
	qILEN-3-3	T11	T574	T5746F06 - T5743D06b	3a/b and 12	-3.56****					17.5	
	qILEN-3-4†	T12	T89	T5741D01a - T5742A10a	16	-2.03****					11.1	
LLS	qLLS-7-1	Joint	T89	CA100438-215 - PAP06F11a	20	-1.91**	+2.66**	NS	NS	-4.35****	3.2	1.2
	qLLS-7-1	TT1	T89	CA100438-215 - PAP06F11a	20	-5.11****					14.8	
	qLLS-7-1	TT2	T89	CA100438-215 - PAP06F11a	20	-10.73****					23.1	
LW	qLW-2-1	T12	T89	TC62694-450 - RZ717	3a/b	-0.13****					12.7	
	qLW-5-1	T12	T574	CA078499-800 - PCD148a	7a-2/b-I and 5a/b	-0.10****					10.7	

[a] Single environment phenotypes identified with prefixes *G* Griffin and *T* Tifton followed by *10* (2010), *11* (2011), *12* (2012) and T1 (1st date) or T2 (2nd date). Joint refers to joint environment analysis.
[b] Linkage group nomenclature following our companion paper.
[c] Additive (and/or dominance) effect of a QTL.
[d] αe at Griffin (2011) for ILEN or Griffin (1st date) for LLS; [e] αe at Tifton (2011) for ILEN or Tifton (1st date) for LLS; [f] αe at Griffin (2012) for ILEN or Griffin (2nd date) for LLS; [g] αe at Tifton (2012) for ILEN or Tifton (2nd date) for LLS.
[h] percent variation explained by 'α' effect; [i] percent variation explained by 'αe' effect.
[j] Novel QTL identified by MCIM (not detected/significant at permutation threshold following CIM).
*0.05, **0.01, ****0.001, *NS* not significant.

Table 4.4 Epistatic effects underlying bermudagrass morphology detected by mixed-model based CIM (MCIM) procedure implemented in QTL Network v. 2.0.

Trait	Env.[a]	Source	Loci_(i)			Loci_(j)			$\alpha\alpha$[d]	$\alpha\alpha e1$[e]	$\alpha\alpha e2$[f]	$\alpha\alpha e3$[g]	$\alpha\alpha e4$[h]	$\alpha\alpha e5$[i]	$R^2_{\alpha\alpha}$ (%)[j]	$R^2_{\alpha\alpha e}$ (%)[k]
			QTL_i[b]	Interval_i	Linkage group[c]	QTL_j	Interval_j	Linkage group								
HT	Joint	T574	qHT-3-3†	CA138897-420 - TC64929-160	3a/b and 12	qHT-4-1†	CA097171-420 - CA097171-280	4a/b	+1.34****	-1.09*	NS	+3.16****	NS	NS	1.1	1.9
	T11	T574	qHT-3-3†	CA138897-420 - TC64929-160	3a/b and 12	qHT-4-1†	CA097171-420 - CA097171-280	4a/b	+4.63****						16.6	
ILEN	Joint	T89	qILEN-6-2†	TC62910-300 - RZ142b	1c/d	qILEN-1-1†	PCD021b - TC48763-210	4c/d-1 and 18	-2.01****	NS	NS	NS	NS		3.4	1.2
		T574	qILEN-6-1	T5745B01 - T5743D12b	6a/b	qILEN-5-1	T5741A08bc - T5745F10b	7a-2/b-I and 5a/b	+2.02****	NS	NS	NS	NS		10.5	1.8
			qILEN-6.3†	T5741C09a - T5741D12	6a/b	qILEN-3-3	T5746F06 - T5743D06b	3a/b and 12	+2.59****	NS	+1.64*	NS	NS			
			qLEN-9-2†	RZ596c - CA216782-120	9 and 11	qILEN-4-1†	PCD032c - T5741F10a	4a/b	+1.99****	NS	NS	NS	NS			
	G12	T574	qILEN-6-1	T5745B01 - T5743D12b	6a/b	qILEN-5-1	T5741A08bc - T5745F10b	7a-2/b-I and 5a/b	+3.64****						18.6	
LLS	Joint	T89	qLLS-6-1†	TC61478-580 - TC61478-600	1c/d	qLLS-4-1†	PCD137c - TC51780-120	6a/b	+7.24****	NS	NS	NS	NS		5.3	1.7
			qLLS-9-3†	T5745D12c - T5748G06ab	2a/b/c	qLLS-7-1	CA100438-215 - PAP06F11a	20	+4.28****	NS	NS	NS	NS			
			qLLS-9-2	RZ588 - T5746C08	1a/b	qLLS-3-1†	RZ538 - TC55445-110	3c/d and 16	+5.05****	NS	NS	NS	NS			
	TT1	T89	qLLS-5-1†	T5742F07 - PAP10A04b	15	qLLS-2-1†	CA097696-140 - T5741F06c	4a/b	-7.13****						17.6	
LLEN	Joint	T89	qLLEN-6-1†	RZ900 - CA294786-340	7a/b and 11	qLLEN-4-1†	TC67261-150/230 - T5745B05	14 and 27	+4.71****	NS	NS				7.2	1.5
		T574	qLLEN-3-2†	TC56094-130 - T5745H10de	3a/b and 12	qLLEN-8-1†	CA094069-210 - T5743B03c	8 and 10	+4.54****	NS	NS				8.6	0.1
LW	Joint	T89	qLW-3-1	CA162523-100 - CA265902-300	16	qLW-3-2	TC48402-320 - RZ543b	12	+0.20****	NS	NS	NS			10.9	0.5
	T11	T574	qLW-5-1	CA078499-800 - PCD148ab	7a-2/b-I and 5a/b	qLW-3-4†	T5743D06a - PAP07C04a	3a/b and 12	+0.27****						18.7	
	G12	T89	qLW-3-1	CA162523-100 - CA265902-300	16	qLW-3-2	TC48402-320 - RZ543b	12	+0.26****						22.5	

[a] Single environment phenotypes identified with prefixes *G* Griffin and *T* Tifton followed by *10* (2010), *11* (2011), *12* (2012) and T1 (1st date) or T2 (2nd date). Joint refers to joint environment analysis.
[b] QTL nomenclature following CIM; novel QTLs are identified with suffix '†'.
[c] Linkage group nomenclature following our companion paper.
[d] Additive (and/or dominance) by additive (and/or dominance) (i.e., $\alpha\alpha$) effect of a QTL.
[efghi] $\alpha\alpha e$ for HT (5 environments), ILEN (4 environments), LLS (4 environments), LLEN (2 environments), and LW (3 environments)
[j] percent variation explained by '$\alpha\alpha$' effect; [k] percent variation explained by '$\alpha\alpha e$' effect.
*0.05, ****0.001, *NS* not significant.

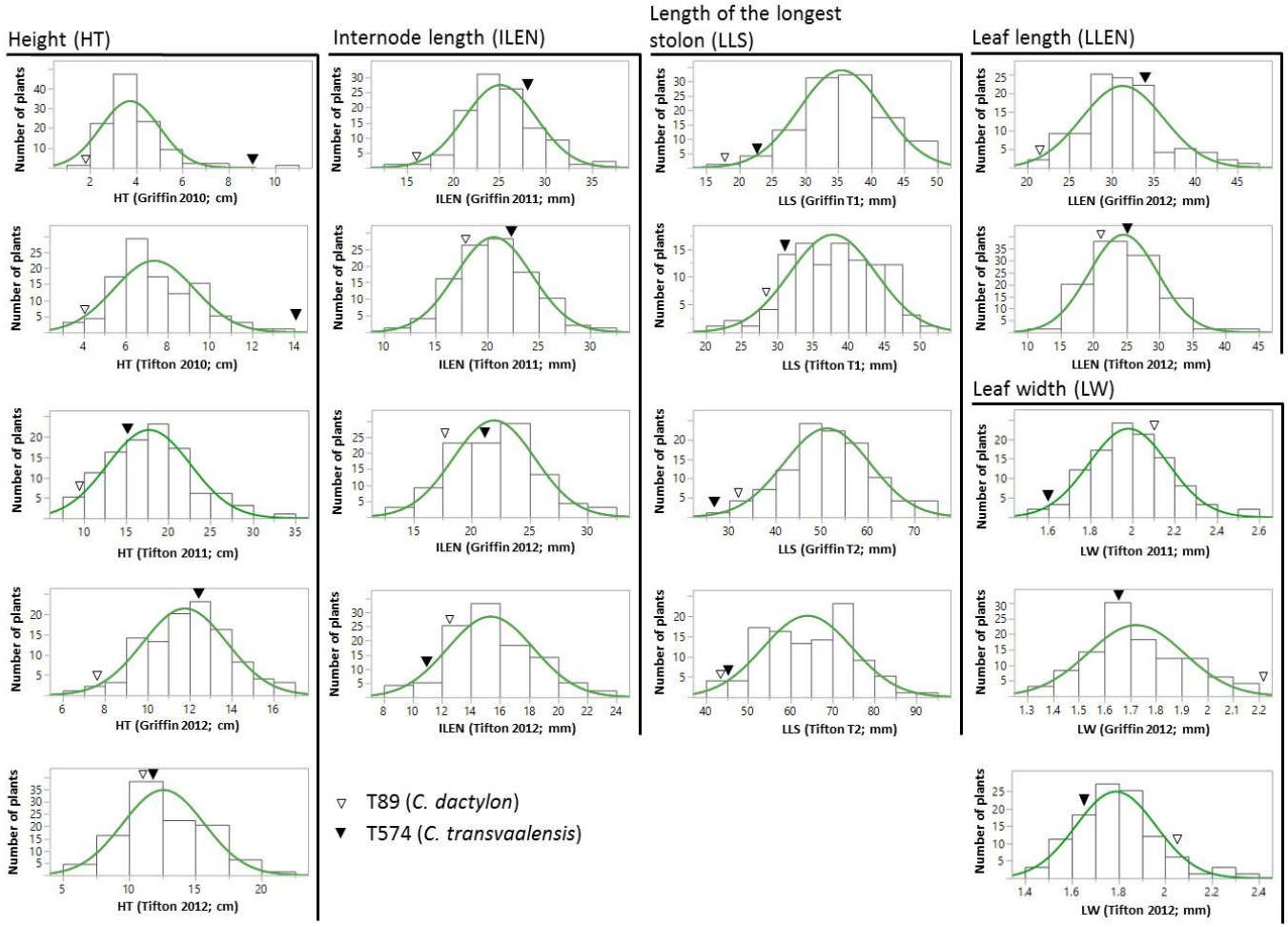
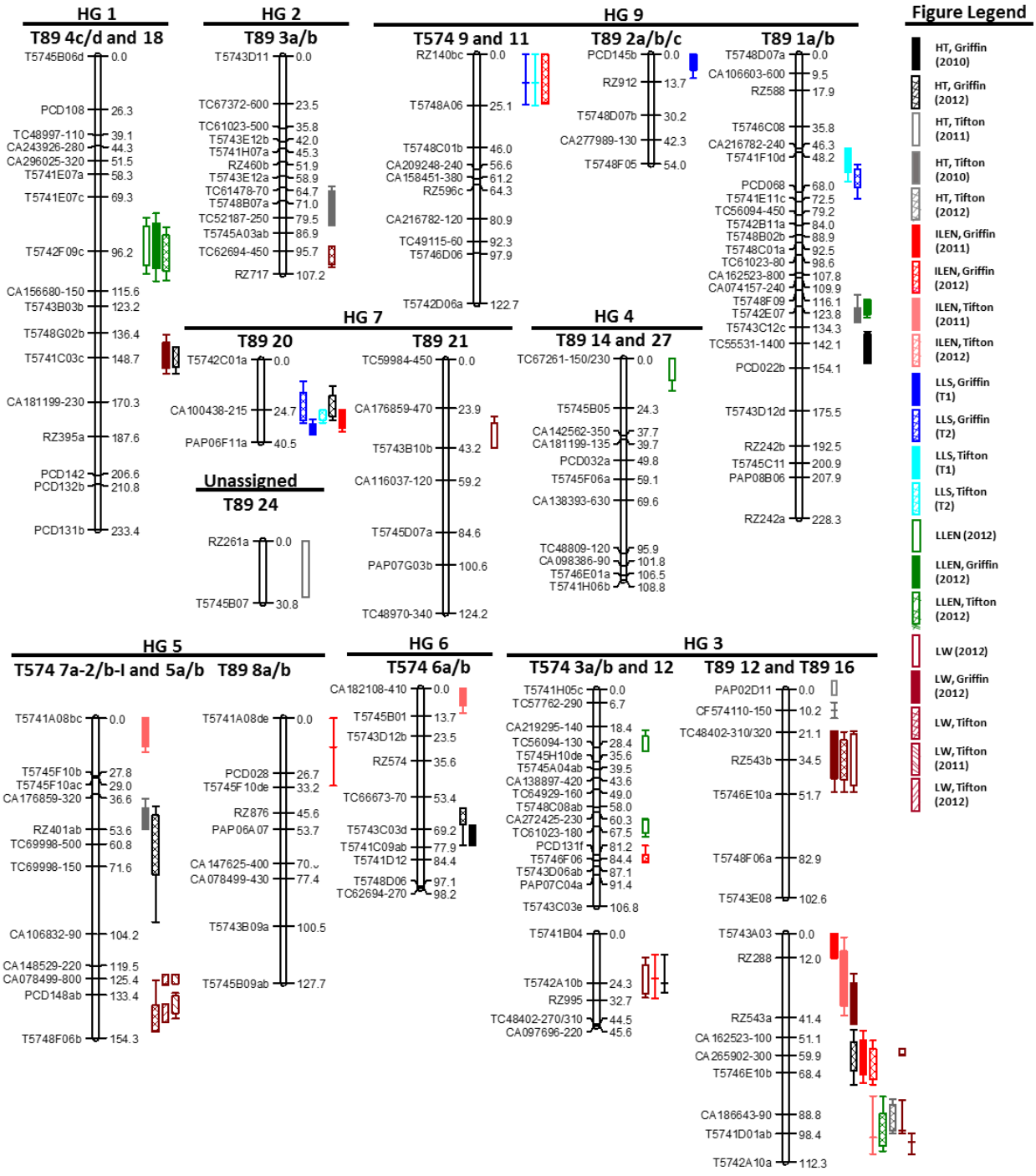


Figure 4.1 Distribution of morphological traits in clonal propagates of *C. dactylon* x *transvaalensis* intraspecific hybrids in different environments. Environments include: locations (i.e., Tifton and Griffin), year (2010, 2011, and 2012), and time (T1 and T2 in 2012 for LLS). Parental means in different environments are marked by *solid* (T574) and *open* (T89) triangles.

Figure 4.2 A bermudagrass genetic linkage map (*left-hand side*) superimposed with candidate genomic regions (*right-hand side*) detected for turf morphological traits. In the parental genetic linkage maps (i.e., *T574* and *T89*), composite interval mapping (CIM) identified a number of significant QTLs (M-QTLs) for plant height (HT), internode length (ILEN), length of the longest stolon (LLS), leaf length (LLEN), and leaf width (LW). QTLs are specified with LOD support intervals identified with *rectangular boxes* (1-LOD) with *whiskers* (2-LOD). Putative homo(eo)logous regions with harboring suggestive QTLs (i.e., LOD as low as 2) are identified using *colored* line segments corresponding to associated M-QTLs and span 2-LOD intervals. Each linkage group is identified with its parental source and assigned homology group (i.e., HG).


HG 1
T89 4c/d and 18
T5745B06d 0.0
PCD108 26.3
TC48997-110 39.1
CA243926-280 44.3
CA296025-320 51.5
T5741E07a 58.3
T5741E07c 69.3
T5742F09c 96.2
CA156680-150 115.6
T5743B03b 123.2
T5748G02b 136.4
T5741C03c 148.7
CA181199-230 170.3
RZ395a 187.6
PCD142 206.6
PCD132b 210.8
PCD131b 233.4
HG 2
T89 3a/b
T5743D11 0.0
TC67372-600 23.5
TC61023-500 35.8
T5743E12b 42.0
T5741H07a 45.3
RZ460b 51.9
T5743E12a 58.9
TC61478-70 64.7
T5748B07a 71.0
TC52187-250 79.5
T5745A03ab 86.9
TC62694-450 95.7
RZ717 107.2
HG 9
T574 9 and 11
RZ140bc 0.0
T5748A06 25.1
T5748C01b 46.0
CA209248-240 56.6
CA158451-380 61.2
RZ596c 64.3
CA216782-120 80.9
TC49115-60 92.3
T5746D06 97.9
T5742D06a 122.7
T89 2a/b/c
PCD145b 0.0
RZ912 13.7
T5748D07b 30.2
CA277989-130 42.3
T5748F05 54.0
T89 1a/b
T5748D07a 0.0
CA106603-600 9.5
RZ588 17.9
T5746C08 35.8
CA216782-240 46.3
T5741F10d 48.2
PCD068 68.0
T5741E11c 72.5
TC56094-450 79.2
T5742B11a 84.0
T5748B02b 88.9
T5748C01a 92.5
TC61023-80 98.6
CA162523-800 107.8
CA074157-240 109.9
T5748F09 116.1
T5742E07 123.8
T5743C12c 134.3
TC55531-1400 142.1
PCD022b 154.1
T5743D12d 175.5
RZ242b 192.5
T5745C11 200.9
PAP08B06 207.9
RZ242a 228.3
HG 7
T89 20
T5742C01a 0.0
CA100438-215 24.7
PAP06F11a 40.5
T89 21
TC59984-450 0.0
CA176859-470 23.9
T5743B10b 43.2
CA116037-120 59.2
T5745D07a 84.6
PAP07G03b 100.6
TC48970-340 124.2
HG 4
T89 14 and 27
TC67261-150/230 0.0
T5745B05 24.3
CA142562-350 37.7
CA181199-135 39.7
PCD032a 49.8
T5745F06a 59.1
CA138393-630 69.6
TC48809-120 95.9
CA098386-90 101.8
T5746E01a 106.5
T5741H06b 108.8
Unassigned
T89 24
RZ261a 0.0
T5745B07 30.8
HG 5
T574 7a-2/b-I and 5a/b T89 8a/b
T5741A08bc 0.0
T5745F10b 27.8
T5745F10ac 29.0
CA176859-320 36.6
RZ401ab 53.6
TC69998-500 60.8
TC69998-150 71.6
CA106832-90 104.2
CA148529-220 119.5
CA078499-800 125.4
PCD148ab 133.4
T5748F06b 154.3
T5741A08de 0.0
PCD028 26.7
T5745F10de 33.2
RZ876 45.6
PAP06A07 53.7
CA147625-400 70.
CA078499-430 77.4
T5743B09a 100.5
T5745B09ab 127.7
HG 6
T574 6a/b
CA182108-410 0.0
T5745B01 13.7
T5743D12b 23.5
RZ574 35.6
TC66673-70 53.4
T5743C03d 69.2
T5741C09ab 77.9
T5741D12 84.4
T5748D06 97.1
TC62694-270 98.2
HG 3
T574 3a/b and 12
T5741H05c 0.0
TC57762-290 6.7
CA219295-140 18.4
TC56094-130 28.4
T5745H10de 35.6
T5745A04ab 39.5
CA138897-420 43.6
TC64929-160 49.0
T5748C08ab 58.0
CA272425-230 60.3
TC61023-180 67.5
PCD131f 81.2
T5746F06 84.4
T5743D06ab 87.1
PAP07C04a 91.4
T5743C03e 106.8
T5741B04 0.0
T5742A10b 24.3
RZ995 32.7
TC48402-270/310 44.5
CA097696-220 45.6
T89 12 and T89 16
PAP02D11 0.0
CF574110-150 10.2
TC48402-310/320 21.1
RZ543b 34.5
T5746E10a 51.7
T5748F06a 82.9
T5743E08 102.6
T5743A03 0.0
RZ288 12.0
RZ543a 41.4
CA162523-100 51.1
CA265902-300 59.9
T5746E10b 68.4
CA186643-90 88.8
T5741D01ab 98.4
T5742A10a 112.3
Figure Legend
HT, Griffin (2010)
HT, Griffin (2012)
HT, Tifton (2011)
HT, Tifton (2010)
HT, Tifton (2012)
ILEN, Griffin (2011)
ILEN, Griffin (2012)
ILEN, Tifton (2011)
ILEN, Tifton (2012)
LLS, Griffin (T1)
LLS, Griffin (T2)
LLS, Tifton (T1)
LLS, Tifton (T2)
LLEN (2012)
LLEN, Griffin (2012)
LLEN, Tifton (2012)
LW (2012)
LW, Griffin (2012)
LW, Tifton
LW, Tifton (2011)
LW, Tifton (2012)

CHAPTER 5

DISSECTING QUANTITATIVE VARIATION INTROGRESSED INTO UPLAND COTTON USING QTL-STACKED SEGREGATING POPULATIONS [4]

[4] Khanal, S, Patel, J.D., Adhikari, J., Chandnani, R., Wang Z., Das S., Brown N., Jones, D., Chee, P.W., and Paterson, A.H. 2018. To be submitted to *Theoretical and Applied Genetics*

Abstract

Mendelizing quantitative trait loci (QTL) and identifying 'stable' QTL are prerequisites for marker-assisted selection in crop improvement. We developed QTL-stacked populations in two elite *G. hirsutum* backgrounds (GA2004230 and R01-40-08, the latter containing a chromosomal segment introgressed from *G. barbadense*) targeting eight QTL alleles introgressed into advanced-backcross *G. hirsutum* lines affecting fiber elongation (three each from *G. tomentosum*, *Gt*; and *G. mustelinum*, *Gm*), fiber fineness (one from *Gt*), and fiber strength (one from *Gm*). A total of twenty-one QTL-stacked F_1 individuals (nine in GA2004230 and 12 in R01-40-08 backgrounds) were selfed to generate F_2 populations segregating at two to three QTL regions with 33 to 132 plants per population (totaling 1,509 plants), which were field tested for fiber quality traits, genotyped at target QTL regions, selectively advanced to $F_{2:3}$ generation and assessed at two different locations (Athens and Tifton GA). Favorable shifts in average phenotypes of selected traits were partially explained by genotypes at introgressed QTL regions, with a subset consistent across backgrounds and/or generations. One-way analysis of variance in F_2 populations validated the effects of two target QTL in both backgrounds (one to three populations each), and four more in at least one background. While three of four *Gm*-QTL allele effects were detected, *Gt*-QTL effects were relatively elusive in current mapping populations. *Gm*-QTL 'qELO-1-1' and 'qELO-11-1' were evidently 'stable' and consistently increased fiber elongation in backcross-selfed populations (prior study) and in QTL-stacked secondary segregating populations (current study). A previously undetected *Gm*-QTL introgressed into chromosome 11 explained a significant proportion of variation in fiber fineness in F_2 and pooled analysis of $F_2/F_{2:3}$ lines in several populations. Compared to average phenotypes of appropriate parental backgrounds, selectively advanced lines conferred fiber quality improvements as high as 23.3-24.0% (242-020 and 239-145) for micronaire, 10.5-11.0% (242-004 and 241-036) for strength, and 28.6-47.6% (234-138 and 244-086) for elongation. Phenotypic differences between singly homozygous and doubly homozygous QTL-stacks were also evident in some populations. Our results attest to the promise of MAS as a molecular breeding tool for simultaneously validating QTL effects and developing QTL-stacked

germplasm in different genetic backgrounds. Validated QTL and QTL-stacked lines with enhanced fiber quality represent valuable breeding resources for developing improved cotton cultivars.

Introduction

Cotton is the most important natural fiber for the modern textile industry, with ever-increasing demand largely sustained by two domesticated tetraploid species viz. *Gossypium hirsutum* L. (AD1) and *G. barbadense* L. (AD2). A burgeoning human population, improvement in living standards, and increasing preference for high-quality textile from renewable sources together with increased efficiency and sophistication of spinning technology (Felker 2001) have renewed interest in increasing fiber quality of cultivated cotton (May 1999; Chee and Campbell 2009). However, commercial breeding and production practices superimposed on a backdrop of several evolutionary bottlenecks have led to severe depletion of genetic variation within the cultivated gene pool (Paterson et al. 2004; Chee and Campbell 2009). Negative correlation between yield components and fiber quality traits also pose a serious challenge towards raising fiber quality to a new 'adaptive peak' (Wright 1968), particularly by conventional breeding approaches.

The genetic dissection of complex traits into underlying quantitative trait loci (QTL) creates opportunities to manipulate quantitative traits based on Mendelian principles, using marker-assisted selection (MAS), QTL pyramiding, and map-based cloning, and has potential to mitigate some of the challenges hindering improvement of cotton fiber quality (Chee and Campbell 2009; Fang 2015). Hundreds of QTL underlying fiber quality characteristics have been described (Said et al. 2013; Said et al. 2015a; Said et al. 2015b), which apparently intersperse in genetic linkage maps derived from crosses between different genetic backgrounds, tested in different environments, and are often tagged with different volumes and classes of genetic markers (Said et al. 2015b). In a recent review (Zhang et al. 2014), the authors could not find any commercial cotton cultivar coming directly out of interspecific introgression breeding between *G. hirsutum* and *G. barbadense*, the most frequent interspecific hybridization attempted for Upland cotton improvement. Stability (i.e., consistency) of QTL expression has been a major concern (Fang 2015).

Nevertheless, some fiber quality QTL show stable and significant genetic effects in different environments and backgrounds, suggesting that QTL-MAS can be used to enhance fiber quality traits in Upland cotton (Paterson et al. 2003; Sun et al. 2012; Wang et al. 2012b; Islam et al. 2014). Successful application of the QTL-MAS in cotton breeding is expected to facilitate simultaneous improvement in lint yield and fiber quality, and to maximize selection gains by facilitating parental selection, reducing phenotyping cost, and expediting generation advance. Both private and public cotton breeding programs are increasingly using molecular breeding strategies to improve cultivated cotton.

For the past two-and-half decades, traders and breeders alike have mostly relied on High Volume Instrument (HVI) fiber data to make marketing and/or selection decisions, particularly focusing on fiber length, strength, and fineness, the three fiber properties with standard calibration measures. Percent bundle elongation at break, commonly called fiber elongation, from HVI analysis is not currently reported in official cotton classing, but recent studies suggest that it could be reliably used in breeding towards simultaneous improvement of rupture properties (fiber tenacity and elongation) (Hequet et al. 2007; Ng et al. 2014b). Fiber elongation measures the degree of stretchability or elasticity of the fibers before a break occurs and is highly correlated with fiber loss during processing (ginning, carding, spinning, and weaving) (Benzina et al. 2007; Faulkner et al. 2012; Hequet et al. 2014). Similarly, HVI micronaire, measured by recording resistance to air permeability of a constant mass of cotton fiber compressed to a fixed volume, has been widely adopted and accepted as a proxy for fiber fineness and maturity.

Breeders have routinely introduced and shuffled exotic alleles from primitive *G. hirsutum* and *G. barbadense* accessions into modern varieties (McCarty et al. 1995; McCarty et al. 2004; McCarty et al. 2007; Zhang et al. 2014), with increasing opportunity for application of molecular genomic tools based on published marker-trait associations (Cao et al. 2014; Said et al. 2015a; Adhikari et al. 2017; Chandnani et al. 2017b; Chandnani et al. 2017c;). Systematic introgressions from *G. tomentosum* Nutall ex Seemann (AD3), *G. mustelinum* Miers ex Watt (AD4), and *G. darwinii* Watt (AD5) have recently been explored

(Waghmare et al. 2005; Zhang et al. 2011; Wang et al. 2012a; Chen et al. 2015; Oluoch et al. 2016) to introduce rich novel allelic variation into the cultivated gene pool. Interspecific crosses between *G. hirsutum* and *G. tomentosum* or *G. mustelinum* have been used for F_2 mapping (HT or HM map) (Waghmare et al. 2005; Wang et al. 2016b), backcross-selfed mating design (Chee et al. 2005b) QTL analysis (Zhang et al. 2011; Wang et al. 2016a; Wang et al. 2016b; Wang et al. 2017a; Wang et al. 2017b;), and chromatin transmission studies (Waghmare et al. 2016; Chandnani et al. 2017a). Despite the extremely poor fiber quality of these wild species, transgressive segregation in fiber quality was traced to favorable alleles from the wild, attesting to assertions that seemingly unfavorable parents could contribute 'stable' QTL with significant phenotypic effects (Paterson et al. 1988; Tanksley et al. 1996). A number of favorable QTL alleles were contributed by *G. tomentosum* and *G. mustelinum* for fiber elongation and fineness – each trait with relatively short history of selection in scientific cotton breeding (Zhang et al. 2011).

Backcross-selfed introgression lines developed from HT and HM studies carry favorable interspecific alleles in a near-isogenic state, for which 'stability' needs to be assessed in different genetic backgrounds, over different generations, and preferably under different environments. The objective of this research is to determine biometric parameters of QTL-MAS employed to assess several *G. tomentosum* and *G. mustelinum* QTL transferred from near-isogenic introgression lines to secondary segregating populations in genetic backgrounds of elite *G. hirsutum*. Specifically, six fiber elongation (qELO-1-1, qELO-11-1, qELO-21-1, qFE11.1, qFE14.1, and qFE21.1), one fiber fineness (qFF07.1), and one fiber strength (qSTR-25-1) QTL allele(s) were targeted for developing 21 QTL-stacked secondary segregating populations (F_2 and $F_{2:3}$) to validate individual and interactive QTL effects and 'stability' in partial genetic backgrounds of GA2004230 and R01-40-08. Several QTL from *G. tomentosum* and *G. mustelinum* offer avenues for rapid selection gains for fiber quality traits.

Materials and Methods

Plant Materials and Population Development

Genetic materials and the population development approach used in generating secondary segregating populations used in the current study is illustrated in **Figure 5.1**. The target QTL, chromosomal affiliations, diagnostic RFLP loci and/or flanking SSR markers, and pertinent QTL information are presented in **Supplemental File S5.01**. In summary, *G. hirsutum* x *G. tomentosum* (HT) advanced-backcross lines (BC_3F_2) carrying a fiber fineness (qFF-7-1) and three fiber elongation QTL (qFE11-1, qFE-14-1, and qFE-21-1) (herein referred to as *Gt*-QTL) were identified from Zhang et al. (2011), retrieved from UGA-PGML (http://www.plantgenome.uga.edu/), and advanced to $BC_3F_{2:3}$ generation in 2011. Selected $BC_3F_{2:3}$ lines with *Gt*-QTL were crossed with two *G. hirsutum* lines viz. GA2004230 (GA230) and R01-40-08 (R01) representing southeastern US elite germplasm developed at the University of Georgia (UGA) Molecular Cotton Breeding Laboratory (www.nespal.org/peng_lab). R01-40-08 is a near isogenic introgression line (NIIL) homozygous for *G. barbadense* cv. Pima S6 chromatin segment carrying a fiber length QTL qFL-chr1, which conditions longer fiber in the recurrent background of *G. hirsutum* cv. Tamcot 2111 (Shen et al. 2011). Further, NIILs from *G. hirsutum* x *G. mustelinum* (HM) advanced-backcross lines ($BC_3F_{2:3}$) carrying a fiber strength (qSTR-25-1) and three fiber elongation QTL (qELO-1-1, qELO-11-1, and qELO-21-1) (herein referred to as *Gm*-QTL) were received from the UGA Molecular Cotton Breeding Lab.

Gt- and *Gm*-QTL selections were planted in crossing blocks at the UGA Plant Science Farm, Watkinsville, GA in 2012, where *Gt*-QTL x *Gm*-QTL crosses within GA230 and R01 backgrounds were made in all possible combinations. Recurrent parents of *Gt*- and *Gm*-QTL selections were different. While *Gt*-QTL in recurrent background of CA3093 were introduced to GA230 and R01, *Gm*-QTL were introgressed in recurrent background of PD94042, another *G. hirsutum* cultivar. To distinguish genetic effects of background interactions (i.e., transgressive segregation) versus true effects of target QTL, GA230

and R01 were also crossed with PD94042 (i.e., parental crosses). First generation hybrids from each cross were grown in a greenhouse using seedling containers filled with commercial garden soil mix. Single F_1 plants from each parental cross were selfed to develop F_2 populations. Diagnostic SSR markers were used to genotype *Gt*-QTL x *Gm*-QTL crosses (20 individuals each when available) at target regions to identify QTL-stacked lines, which were transferred to bigger pots and selfed to develop F_2 populations. A few F_2 lines from each population were selectively advanced to $F_{2:3}$ generation based on genotypes at diagnostic marker loci. Specifically, individuals homozygous at one or more putative QTL were advanced to multilocation replicated trials (discussed later). F_2 and $F_{2:3}$ lines in partial genetic backgrounds of GA230 and R01 were collectively referred to as GA230- or R01-populations, respectively.

Marker Resources

Gt-QTL targets were originally mapped and delineated with diagnostic RFLP markers (Zhang et al. 2011) (**Supplemental File S5.01**). Since the HT map was constructed from a subset of RFLPs used in a composite HB map (Rong et al. 2004), heterologous bridge loci including orthologs of diagnostic RFLPs could be traced in the HB map, which then was juxtaposed with SSR-rich maps [HM map (Wang et al. 2016b), HB maps (Guo et al. 2007; Yu et al. 2007), and a comprehensive reference map (Yu et al. 2010)]. Markers potentially spanning *Gt*-QTL targets were identified, and oligonucleotide primers [synthesized by Eurofins MWG Operon (Huntsville, AL)] were used to screen *G. hirsutum* (var. CA3093) and *G. tomentosum* (acc. AD3-16) for polymorphisms. Polymorphic markers were further screened against a small subset of the HT population to ascertain linkage with diagnostic RFLP loci and putatively linked SSRs were used for marker-assisted selection (MAS) during population development. Accordingly, SSRs spanning target *Gm*-QTL (**Supplemental File S5.01**) and several physically linked markers selected from Wang et al. (2013) were used to develop a subset of informative markers for MAS of *Gm*-QTL in advanced generations. Markers reportedly spanning qFL-chr1 (Shen et al. 2011) were used to track *G. barbadense* alleles.

Segregation ratios at diagnostic marker loci [i.e., 1:2:1 (two homozygotes and a heterozygote) for a codominant marker and 3:1 (presence vs absence) for a dominant marker] and deviations from independent assortment of alleles at two unlinked loci were tested for the goodness of fit using Pearson's chi squared tests ($P = 0.01$).

Wet Lab Protocols

Genomic DNA was extracted using a modified cetylmethylammonium bromide (CTAB) method (Paterson et al. 1993). A single, unfolded, and preferably second true leaf was collected from individual plants early in the morning. An Omni Bead Ruptor 24 Homogenizer (Omni International) was used for grinding the leaf tissues, and nuclei extraction and nuclei lysis buffer were added as a 1:1 solution. Downstream procedures followed the established protocol with an adjustment in volumes of added solutions for the mini-prep. The quality and quantity of genomic DNA was assessed with a NanoDrop 2000 (Thermo Scientific). PCR reactions and procedures to resolve amplified fragments followed our standard protocols (Khanal et al. 2017). Some genotyping involved fluorescently labelled primers, modified PCR protocol, and analysis using an ABI 3730 automatic DNA Analyser (available upon request).

Field Trials and Phenotyping

QTL-stacked F_2 populations, two parental F_2 populations, and 5 parental replicates (in 1m plots) were hand planted at the Plant Science Farm, Watkinsville, GA in 2013. Agronomic practices including plant spacing, irrigation, fertilization, and pest control followed commercial recommendations. Fiber samples were hand-picked from 15 open bolls per plant (when available). Samples were ginned using an 8-saw gin and 10 grams of lint were sent to the Cotton Incorporated Textile Services Laboratory (Cotton Inc., Cary, NC) where six standard fiber quality parameters including fiber fineness or micronaire (MIC), upper half mean fiber length (UHM), fiber length uniformity (UI), fiber strength (STR), fiber elongation (ELO), and short fiber content (SFC%) were determined using a High-Volume Precision Instrument (HVI; Zellweger-Uster, Knoxville, Tenn.). Populations with poor stands (due to poor germination) and/or late

maturity were replanted at the same location in 2014, and fiber properties were determined as discussed above.

Selected $F_{2:3}$ lines and parental replicates were hand planted in 1m plots (spaced 1m apart) in randomized complete blocks (2 replicates) at two locations (Plant Science Farm at Watkinsville, GA; and Gibbs farm, Tifton, GA) in 2014. A total of 25 boll samples were hand-harvested from each plot, ginned using a 12-saw gin, and fiber phenotype (HVI) for standard fiber quality parameters were obtained from HVI analysis at Cotton Inc.

Phenotypic Analysis of F_2 and $F_{2:3}$ Trials

JMP Pro was used to compute population distributions statistics (mean, standard deviations) and Pearson's correlation coefficients, and to perform ANOVA (one-way and two-way) and mean comparison procedures. Specifically, LSD test employing Student's t-tests, Tukey-Kramer HSD, and With Control, Dunnett's tests (against parental controls) were executed with significance declared at alpha level of 0.05. Partitioning of phenotypic variances into genotype (G), environment (E), and genotype x environment (G x E) interactions among parental lines, selected F_2 individuals, and their $F_{2:3}$ progenies were carried out using standard least squares models that perform two-way ANOVAs, with significance declared at $p < 0.0001$.

Marker-trait Associations at Diagnostic SSR loci

JMP Pro was used to identify marker-trait associations (Student's *t*-test, ANOVA). Single-comparison confidence level (CL) of 99% ($p < 0.01$) was used, while 95% CL ($p < 0.05$) were also reported with consideration that our inferences were primarily based on genotypes at few predetermined marker-tagged QTL-introgressed sites and that observed associations were further assessed among different populations across two genetic backgrounds, and in $F_{2:3}$ multilocational trials. Hence, nominal associations (i.e., $0.01 > p < 0.05$) were discussed to evaluate stability and replicability of potential QTL. The percentage of total phenotypic variance explained (R^2), QTL effects (additive effect of an allele substitution), and

significance of digenic interactions (i.e., haplotype at two loci influencing response trait phenotypes) were obtained from JMP Pro. Gene action (additivity and dominance) was computed as described (Paterson et al. 1990).

For loci segregating in two or more populations of one background, or in both backgrounds, two-way ANOVA was performed using the standard least squares 'Fit Model' function employed in JMP Pro. The analytical model included genotype (G), family (F) and genotype x family (G x F) interaction or background (B) and genotype x background (G x B) interaction as fixed factors. Genotypic effects and interaction effects were considered significant at $p < 0.005$. Similarly, effect of QTL-stacking was assessed based on the 'Fit model' function to test for the significance of main-effects and interactions at diagnostic SSR marker loci, with significance declared at $p < 0.005$ (also reported at $p < 0.05$). Effects of QTL-stacking were further assessed based on ad hoc means comparisons among $F_2/F_{2:3}$ lines and *G. hirsutum* parents in their immediate pedigrees, using pooled phenotypes over generations and locations for different genotypic classes and parental samples.

Results

MAS towards QTL-stacked Populations

SSR markers were exclusively used for identifying advanced-backcross materials carrying introgressed QTL and for MAS to develop QTL-stacked populations. Four *Gt*-QTL were tagged with one to three SSR markers each, whose veracity was supported by genetic linkage to diagnostic RFLPs and/or reported allele size information. Similarly, one to six SSR markers spanning each *Gm*-QTL were identified and used in MAS during population development and QTL analyses (discussed later).

Of a total of 28 possible QTL-stacks (excluding qFE11.1 x qELO-11-1 and qFE21.1 x qELO-21-1) among *Gt*-QTL x *Gm*-QTL in GA230- and R01-populations, 21 combinations carried 23 target QTL-stacks. For the remaining populations, doubly heterozygous individuals were not recovered among the small number of segregating individuals (poor germination). Nonetheless, each target QTL was represented in

two to four GA230- and R01-populations each. The *Gb*-allele spanning qFL-chr1 was stacked in five R01-populations. Individuals heterozygous at target QTL were selfed to constitute F_2 populations for QTL mapping in early segregating generations. A total of 21 populations were grown in 2013 while seven with enough seeds for population establishment were replanted in 2014. *Gm*-QTL selections and their crosses were late in flowering and boll maturation and some populations also showed aberrant phenotypes with few individuals producing enough fiber (10 gm) for HVI analysis (discussed later). Selected F_2 lines from a total of fourteen populations from 2013 were advanced to $F_{2:3}$ generation and evaluated in 2014. Details of QTL mapping populations are presented in **Table 5.1**.

Alleles at the diagnostic loci mostly exhibited normal segregation in F_2 populations, with only two cases showing significant ($P < 0.01$) distortions [i.e., NAU2238/NAU2565/NAU2679 (chromosome 25) in pop. 04 and NAU2238 (chromosome 25) in pop. 13], wherein dominant *Gm*-introgression(s) in chromosome 25 were underrepresented (i.e., 1.5:1 instead of 3:1 presence vs absence; data not shown). The distortions caused significant deviations from expected ratio (i.e., 9:3:3:1 of dominant-green, dominant-white, null-green, and null-white, respectively) of independent assortment of alleles of chromosome 25 markers (dominant or null) and fuzz color (green or white) locus (chromosome 11) in pop. 04 and pop. 15, wherein null-green were overrepresented (needs further assessment in larger populations).

Phenotypic Distribution and Means Comparison

The distribution of fiber quality characteristics in F_2 individuals of GA230- and R01-populations and corresponding distribution in control crosses (i.e., R01 x PD94042 and GA230 x PD94042) is shown in **Table 5.1**, **Figure 5.2,** and **Supplemental File S5.02**. F_2 populations and $F_{2:3}$ selections displayed wider distributions of phenotypes than control crosses, with individuals in both backgrounds showing improvement over parental genotypes in both years (2013 and 2014) and generations. Analysis of variance showed significant differences ($p < 0.0005$) among F_2 and parental means for MIC, UHM, STR, and ELO.

Mean UI and SFC% were significantly different in both backgrounds in 2013, while differences among GA230-populations for those traits were not significant in 2014 (**Supplemental File S5.03**).

Genotype (G) contributed significantly ($p < 0.0001$) to fiber quality variations among parental controls, selected F_2 lines, and their $F_{2:3}$ progenies, in both backgrounds (**Supplemental File S5.04**). While environments (E) showed significant variation, G x E interactions were not significant (at $p < 0.0001$), allowing us to pool data from different years. Genotype contributions to fiber quality phenotypes ranged from 39% (UI and SFC%) to 68% (ELO) in GA230 background, and 26% (SFC%) to 60% (UHM) in R01.

Background-specific means of F_2 populations and selected F_2/$F_{2:3}$ lines compared to the controls (i.e., parents and/or parental crosses) showed a shift towards favorable values for MIC, STR, and ELO - the three traits for which QTL were introduced. On the contrary, there was a lack of favorable shift and even significant decline in UHM, UI, and SFC%. Partial summary of trait specific phenotypic analyses follows:

Micronaire (MIC)

One of the GA230-populations (pop. 15) had significantly lower (improved) MIC ($p < 0.0001$) than GA230 and parental cross GA230 x PD94042. In pooled analysis, selected F_2 and $F_{2:3}$ progenies from pop. 15 had significantly lower MIC values across three environments. Similarly, four different R01-populations (i.e., pop. 04, pop. 11, pop. 13, and pop. 14) had significantly lower MIC values than R01 and R01 x PD94042, and phenotypic values in selected $F_{2:3}$ lines from three of these populations (pop. 04, pop. 11, and pop. 13) were also significantly lower in combined analysis. Average MIC of F_2 populations corresponding to pop. 01 were also significantly lower, both in 2013 and 2014.

Fiber Strength (STR)

Although average STR of most F_2 populations and $F_{2:3}$ selections in both backgrounds were significantly lower than their respective parental controls, one of the R01-populations (pop. 13) had

significantly higher (improved) average STR. Accordingly, 241-036 and 241-039 (two selections from pop. 13) and 242-004 (a selection from pop. 15) showed significantly higher STR values in pooled analysis.

Fiber Elongation (ELO)

Two GA230-populations (i.e., pop. 07 and pop. 19) had significantly higher average ELO values than the GA230 control and GA230 x PD94042 cross, while pop. 8, pop. 10, and pop. 21 were marginally significant ($p < 0.05$). Several selections from pop. 07 and pop. 19 showed significantly higher ELO in pooled analysis of selected lines. In R01 background, pop. 18 had significantly higher average ELO, while pop. 3 was significant both in 2013 and 2014. Further, pop 01 (2013) and pop. 02 showed marginal significance ($p < 0.05$) for higher ELO compared to R01 checks. In pooled analysis, selections from pop. 02, pop. 18, and pop. 20 showed significantly higher ELO values compared to the parental lines.

Fiber length (UHM), length uniformity (UI), and short fiber content (SFC%)

Mean UHM and UI in GA230-populations and $F_{2:3}$ selections were significantly lower (inferior) than the GA230 parent. Several R01-populations showed comparable UHM values to the background controls, but average lengths were significantly lower in $F_{2:3}$ selections. Average UI of most F_2 populations and $F_{2:3}$ selections in both backgrounds were significantly lower than their respective parental controls, while average SFC% were not significantly different. However, several pop. 15 individuals and their $F_{2:3}$ selections had significantly higher SFC% (inferior).

Correlation between Traits

Significant correlations among fiber quality traits corroborate frequently reported associations; specifically, MIC -- ELO and UHM -- STR were positively correlated, while traits across groups (e.g., MIC or ELO against UHM or STR) showed negative associations - the trend was apparent in F_2 and $F_{2:3}$ both in combined and in separate background (GA230 and R01) analyses (**Supplemental File S5.05**). While individual populations, including parental crosses (checks), showed this general pattern, notable exceptions

were evident in four F_2 populations (discussed later). UI and SFC showed consistent negative correlations, with the former positively correlated with UHM and STR, while the latter negatively correlated with other fiber quality traits (i.e., improvement in fiber quality reduces short fiber content). Similarly, correspondence between F_2 and $F_{2:3}$ for MIC, UHM, STR, and ELO were positive and significant ($r > 0.6$, $p < 0.001$) in combined as well as separate analyses of GA230- and R01-populations, while UI and SFC showed weak positive correlations between generations.

Marker-trait Associations at Diagnostic SSR Loci

A summary of marker-trait associations in background-specific populations for MIC, STR, and ELO is presented in **Table 5.2** and those for UHM, UI, and SFC% are reported in **Supplemental File S5.06**. Exploratory marker-trait associations among selected F_2 and $F_{2:3}$ lines are presented in **Supplemental File S5.07**. Partial summary of QTL-specific observations from the current study follows:

Gm-QTL qELO-1-1

NIIL B15-70 carried an ELO QTL (qELO-1-1) mapped to chromosome 1 in recurrent background of a *G. hirsutum* cultivar (PD94042). Diagnostic codominant SSR marker NAU2095 was used for MAS during population development. Seven populations (three GA230- and four R01-populations) with *Gm*-introgressions at target QTL were studied. QTL mapping in four F_2 populations (pop. 12, pop. 18, pop. 19 and pop. 20) was done based on 2013 field trials, while their $F_{2:3}$ selections were evaluated in 2014. Two F_2 populations (pop. 08 and pop. 21) were grown in 2014, while another F_2 population (pop. 03) was grown in 2013 and 2014.

NAU2095 showed significant marker-ELO association in one population each in R01 (pop. 18) and GA230 (pop. 21) and approached significance ($p \leq 0.05$) in pop. 03 (R01-population; 2014) and pop. 08 (GA230-population), with *Gm*-alleles increasing ELO in all four populations. Negative additive effect at NAU2095 is consistent with prior characterization, where *Gm*-alleles increased fiber elongation (Wang

et al. 2016b). F_2 and $F_{2:3}$ selections of pop. 12, pop. 18, pop. 19, and pop. 20 carrying *Gm*-alleles also showed higher ELO (5.1-48.5%) relative to the parental controls (**Figure 5.3a, 5.3b, 5.3c**).

Gm-QTL qELO-11-1 (also, EL11.1) and qSTR-11-1

NIIL B16-78 carried an ELO QTL (qELO-11-1) and a STR QTL (qSTR-11-1) mapped to chromosome 11 in recurrent background of a *G. hirsutum* cultivar (PD94042). While favorable effects for ELO were associated with *Gm*-introgression, *Gh*-alleles were associated with increased STR. Diagnostic SSR markers MUSS123 and NAU3377 were used for MAS during population development. Four populations (two GA230- and two R01-populations) with *Gm*-introgression at target QTL were studied. QTL mapping in three F_2 populations (pop. 02, pop. 07, and pop. 10) was done in 2013, while their $F_{2:3}$ selections were evaluated in 2014. One F_2 population (pop. 09) was grown in 2014.

In single marker genetic analysis, one additional codominant SSR marker near target QTL region (i.e., NAU3341) showed significant marker-elongation associations in two populations of R01 (pop. 02 and pop. 09) and one population of GA230 (pop. 10). *Gm*-alleles of MUSS123 were also associated with fiber elongation in the three populations, while those of NAU3377 were associated with two (pop. 02 and pop. 10). Interval mapping detected significant QTL peaks in the region flanked by MUSS123 and NAU3341, which explained 28-29% of phenotypic variation in each of the three populations (**Table 5.3**). Negative additive effect at the QTL region is consistent with prior characterization, where *Gm*-alleles increased fiber elongation (Wang et al. 2016a; Wang et al. 2016b). The QTL was not detected in pop. 07 (discussed later). The effect of qSTR-11-1 was detected in pop. 02 and in selected $F_2/F_{2:3}$ lines, where *Gh*-alleles increased fiber strength.

In combined analysis of $F_2/F_{2:3}$ selections of pop. 02, we observed an increase of 15-23% in fiber elongation in lines carrying homozygous *Gm*-alleles versus homozygous *Gh*-alleles at chromosome 11 loci (**Figure 5.3d**). Consistent with F_2 observation, selected pop. 07 lines also displayed increased ELO in

combined analysis (**Figure 5.3e**); however, pop. 10 selections were not significantly different from the parental checks (**Figure 5.3f**).

Gm-QTL qELO-21-1 (also, EL21-2) and qUHM-21-1

NIIL B16-78 carried *Gm*-QTL (qELO-21-1 and qUHM-21-1) introgressed in chromosome 21 in recurrent *G. hirsutum* (PD94042) background. While favorable effects for ELO was associated with *Gm*-introgression, *Gh*-alleles were associated with increased UHM. BNL1034 and NAU3074 were used for MAS during population development. Six independent QTL-stacked populations were developed (three GA230-populations and three R01-populations), five (pop. 02, pop. 05, pop. 07, pop. 14, and pop. 16) of which were grown in 2013 and their $F_{2:3}$ selections were evaluated in 2014. One F_2 population (pop. 17) was grown in 2014. Targeted association with ELO was not detected for *Gm* chromosome 21 markers in the current study. Instead, *Gm*-alleles at NAU3074 were associated with a decrease in UHM and UI in pop. 02, while those of NAU3074 and BNL1034 were also associated with a decrease in UHM in pop. 14.

Gm-QTL qSTR-25-1, qMIC-25-1, qUHM-25-1, and EL25.1

B17-008 NIIL was selected for *Gm*-QTL introgression in chromosome 25 mapping colocalized QTL qSTR-25-1, qMIC-25-1, qUHM-25-1, and EL25.1, where favorable effects in phenotypes were associated with *Gm*-introgressions. SSR markers NAU3306, NAU2679, NAU2238, and NAU2565 were used for MAS during population development. Six populations (four in R01 and two in GA230) were studied (pop. 01, pop. 04, pop. 06, pop. 11, pop. 13, pop. 15) of which *Gm*-derived dominant SSRs in the region (NAU2565, NAU2679) showed significant marker-strength association in one population (pop. 13; $p<0.001$ and $R^2=16\%$). In pop. 04, *Gm*-alleles of the diagnostic markers were associated with an increase in fiber length ($p<0.0027$ and $R^2=13\%$), while those of another marker in the region (i.e., NAU2238) were associated with a decrease in MIC ($p<0.0103$ and $R^2=6\%$).

In combined analysis of F_2 and $F_{2:3}$ selections of *Gm*-QTL introgressed in chromosome 25, selected lines from pop. 13 carrying *Gm*-allele showed increased strength (**Figure 5.3l**), which is consistent with F_2

observation. Further, selections of pop. 15 showed a decrease of 11% in MIC in lines carrying *Gm*-alleles, while all selected pop. 13 lines (four in total) carried *Gm*-alleles and showed significantly lower average MIC values (**Figure 5.3j**). A selection each from pop. 11 (239-145) and pop. 04 (235-086) had significantly lower MIC, while two pop. 13 selections (241-036 and 241-039) and a pop. 15 selection (242-004) showed increased fiber strength in the combined analysis.

Gt-QTL qFF07.1

NAU2432 was linked to diagnostic RFLP locus G115bE3C for fiber fineness QTL (qFF07.1) introgressed from *G. tomentosum* into chromosome 7 of *G. hirsutum* cultivar CA3093. BC_3F_3 individuals from line 93-3-2 showed *Gt*-introgression at the locus and were used in MAS for developing six populations (pop. 04, pop. 07, pop. 09, pop. 17, pop. 18, and pop. 19), of which two are GA230- and four R01-populations. Of the populations segregating at the diagnostic SSR locus, NAU2432 showed decreased MIC attributable to the *Gto*-allele in one population each in GA230- (pop. 19) and R01-populations (pop. 04). Combined analysis of $F_2/F_{2:3}$ provided support for the observed marker-trait association in pop. 04, but failed to do so for pop. 19 (**Figure 5.3g, 5.3i**).

Gt-alleles at NAU2432 were also associated with increased ELO in pop. 18 and approached significance ($p < 0.0250$) for increased ELO in pop. 19 (data not shown). In pooled analysis of $F_2/F_{2:3}$ selections, the *Gt*-allele at NAU2432 showed significant overall marker effect ($p < 0.0025$) of increasing ELO, while its main-effect was significant in pop. 4 ($p < 0.0005$) where homozygous *Gt*-alleles increased elongation by ~12% over homozygous *Gh*-alleles. Pooled analysis provided additional support for the *Gt*-allele increasing ELO in pop. 04, pop. 07, pop. 18, and pop. 19 (**Supplemental Table S5.04, Figure 5.3b, 5.3e, 5.3g**).

Gt-QTL qFE11.1

A fiber elongation QTL on chromosome 11 (qFE11.1) was tagged with three SSR markers viz. NAU1014, NAU5212, and BNL2632 based on their linkages with the diagnostic RFLP locus

pBAM422yE3C. BC_3F_3 individuals from line 93-3-5 showed *Gt*-introgression at the locus and were used in MAS for developing six populations (pop. 13, pop. 14, pop. 15, pop. 16, pop. 20, and pop. 21), of which three are GA230- and three R01-populations. In the current study, dominance effect at diagnostic SSR locus NAU1014 increased ELO in pop. 21 (GA230 background), while its effects on ELO was not detected in other populations. However, *Gt*-alleles from NAU1014 also showed significant among family genotypic effect in decreasing MIC. Two F_2 populations (pop. 15 and pop. 21) showed reduced MIC values attributed to *Gt*-allele at NAU1014 locus. In combined analysis of $F_2/F_{2:3}$ selections of pop. 15, homozygous *Gt*-alleles at NAU1014 decreased MIC by ~9% over homozygous *Gm*-alleles.

Gt-QTL qFE14.1, qFF14.1, and qFU14.1

We identified amplicons corresponding to a codominant marker NAU5027 that was linked to diagnostic RFLP locus (pAR815E3C) associated with qFE14.1, qFF14.1, and qFU14.1. Increase in ELO was conditioned by *Gt*-alleles, while the locus showed significant genotype x family (G x F) interaction for fiber fineness and uniformity. Lines from 93-3-10 were used to develop five segregating populations [two GA230- (pop. 05 and pop. 08) and three R01-populations (pop. 01, pop. 02, pop. 03)] following QTL-MAS. In association analysis, NAU5027 showed marginal significance ($p < 0.05$) for increased ELO attributed by *Gt*-allele in pop. 01 (2014). Further, individuals homozygous for *Gt*-alleles at NAU5027 had ~15% increased fiber elongation compared to *Gh*-homozygotes in pooled analysis of $F_2/F_{2:3}$ selections of pop. 02. *G. tomentosum* alleles of NAU5027 were also associated with an increase in UI in pop. 02. Also, suggestive main-effect ($p < 0.05$) at the locus increased both ELO and MIC in pop. 01 (2014).

Gt-QTL qFE21.1 and qFF21.1

Three markers viz. NAU2998, NAU3074, and NAU3731 were linked to the diagnostic RFLP loci G1261aE3C associated with a fiber elongation (qFE21.1) and a fiber fineness QTL (qFF21.1), where the *Gt*-allele mapped to chromosome 21 contributed favorable phenotypic effects. Using lines from 93-3-10 with introgressions at the QTL region, four secondary QTL mapping populations (two each in GA230- and

R01-populations) were developed. Of the four QTL-stacked populations, pop. 10, pop. 11, and pop. 12 were grown in 2013 and pop. 06 in 2014.

In current study, *Gt*-allele from the diagnostic SSR markers were not associated with increased fiber elongation. However, *Gt*-alleles decreased MIC in pop. 12, which is consistent with the fact that qFF21.1, a MIC QTL where *Gt*-allele increases fiber fineness, also maps to the same QTL region. However, in pooled analysis of $F_2/F_{2:3}$ selections of pop. 11, homozygous *Gt*-alleles evidently increased MIC by 17% and decreased SFC% by 4% over homozygous *Gh*-alleles.

Gb-QTL qFL-chr1

A total of five populations carried the *Gb*-allele of marker JESPR56, which reportedly showed significant phenotypic effects associated with the fiber length QTL qFL-chr1 (Shen et al. 2011). Although marker-trait associations were not detected in F_2 populations, average lengths of pop. 01 (2014) and pop. 03 (2014) were not significantly different from R01 parental checks. Contrasts between selectively advanced $F_{2:3}$ genotypes were not significantly different.

Gm-locus for green fuzz phenotype

NIIL B17-008 (selected for *Gm*-QTL qSTR-25-1) was segregating for vibrant green fuzz color, which, based on published literature, maps to chromosome 11 (Dighe et al. 2009). Evidently, the NIIL carried *Gm*-introgression in chromosome 11, as supported by the presence of *Gm*-allele at SSR locus NAU1014 and corresponds to the region that maps fiber elongation QTL in HT and HM. In the current study, green fuzz character inherited from *Gm*-parent was associated with a decrease in MIC; although, significance of these associations ranged widely ($p < 0.43$ to 0.0005), general direction of effect was consistent across populations, suggesting that green fuzz is linked with MIC determinants (**Table 5.2**). Specifically, fuzz color locus was significantly associated with MIC in pop. 01 (in 2013; $p < 0.0005$), pop. 11 ($p < 0.0074$), and pop. 15 ($p < 0.0048$). We also had an opportunity to assess marker-trait associations with NAU1014 alleles from *Gh*, *Gt*, and *Gm*. Significantly lower MIC of pop. 15 and of selected $F_2/F_{2:3}$

lines were partially explained by significant additive effect of *Gt*-allele at NAU1014, which was further supported by 9% lower MIC for genotypes homozygous for *Gt*-introgressions compared to genotypes homozygous for *Gm*-introgressions at NAU1014 among selected F_2 and $F_{2:3}$ from pop. 15, an observation corresponding to 6.5% lower MIC of green/mixed versus white fuzz phenotypes (**Figure 5.3j, 5.3k**). Similarly, combined analysis of selected $F_2/F_{2:3}$ lines of pop. 11 showed that lines carrying homozygous *Gm*-alleles at NAU1014 had 19% lower MIC than heterozygous lines ($p<0.0001$), an observation corresponding to 17% lower MIC of green/mixed versus white fuzz phenotypes (**Figure 5.3j, 5.3k**).

Consistency of QTL Across Populations

A total of 174 two-way ANOVAs were performed for diagnostic SSR loci segregating in two or more F_2 populations to decipher marker-trait associations among background-specific populations to assess their consistency. A significant ($p < 0.005$) among-family genotype effect was detected at 11 (6.3% of cases) marker loci, five in GA230- and six in R01-populations (**Supplemental Table S5.08**). The loci represent four nonoverlapping genomic regions, two in chromosome 11 and one each in chromosomes 1 and 21. Further, fuzz color phenotypic locus showed significant genotype effect for MIC among R01-populations. Accordingly, one significant among-family genotype effect was found for each fiber quality trait except SFC% in GA230 and UI and SFC% in R01-populations. Consistent with F_2 observation, pooled analysis of $F_2/F_{2:3}$ lines showed significant among-population genotypic effects (G) of fuzz color locus in R01-populations for MIC (**Supplemental Table S5.09**). Accordingly, significant G effect were found for an increase in STR in R01-selections carrying *Gh*-alleles at NAU3377, while increase in ELO in selections carrying *Gt*-alleles at the adjacent marker locus (i.e., NAU3341).

Significant G x F interactions were observed at four marker loci (2.3% of cases), indicating different effect of *Gm*-alleles in segregating populations; however, extreme cases of contrasting phenotypic effects were not observed (**Supplemental Table S5.10**).

Effects of QTL Stacking

A total of 816 two-way ANOVAs were performed to detect main-effects of stacked marker loci and their interactions (digenic). A total of 17 of 426 (i.e., 4%) non-redundant digenic models (i.e., single marker per target genomic region) were significant ($p < 0.005$) including five constituting significant interactions. For digenic models without interactions, 12 were significant at $p < 0.005$ and an additional three were significant at $p < 0.05$ (**Table 5.2, Supplemental Table S5.06**). Four models (out of 15) showed favorable shifts in fiber phenotypes attributable to stacked introgressed alleles and includes two each conditioning decreased MIC (i.e., increased fineness) and increased ELO (elongation) in four different populations (**Table 5.2**). Several significant marker-trait associations in F_2 were also detected in pooled analysis of $F_2/F_{2:3}$ (**Table 5.2, Supplemental Table S5.06 and S5.09**), while two significant digenic models in F_2 (pop. 11 and pop. 15) (**Table 5.2**) were consistent in $F_{2:3}$ (data not shown).

Further, ad hoc means comparison (see 'Materials and Methods') was employed to quantify effects of QTL-stacking using 480 digenic combinations of stacked alleles in $F_2/F_{2:3}$ selections from 14 populations. A total of 28 combinations showed stacking effects, where 18 (9 nonredundant) were favorable and 10 (6 nonredundant) were unfavorable. For example, pop. 18 (R01 background) and pop. 19 (GA230 background) were segregating at two codominant marker loci viz. NAU2432 (*Gt*-QTL) and NAU2095 (*Gm*-QTL), both of which showed significant main effect for ELO in pop. 18. Three lines were selected from each population to constitute $F_{2:3}$ generation based on marker genotypes. Two of the lines were homozygous for exotic alleles (i.e., *G. tomentosum* or *G. mustelinum*) at one or the other locus and the third was homozygous at both marker loci. Phenotypes of each genotypic class in selected F_2 lines (Athens, 2013) and $F_{2:3}$ plots (replicated twice at Athens and Tifton in 2014) of pop. 18 and pop. 19 were pooled and compared against CA3093, PD942, and one of the two background populations (i.e., GA230 or R01) (**Figure 5.3b**). In pop. 18, doubly homozygous lines showed 22% to 29% higher ELO over parental lines, while those in pop. 19 showed 36% to 48% higher ELO. Further, singly homozygous lines for NAU2432

(*Gt*-introgression) showed relatively little increase in ELO (3-9%) in pop. 18 compared to 24-35% increase in pop. 19. Also, singly homozygous lines for NAU2095 (*Gm*-introgression) showed plausible increase in elongation in both populations (19-26% in pop. 18 and 11-21% in pop. 19), suggesting that qELO-1-1 from *G. mustelinum* displayed stable effects in different genetic backgrounds.

Accordingly, favorable stacking effects were detected in seven different populations and included two cases of enhanced MIC (increased fineness), one of increased STR (strength) and six cases of increased ELO (elongation). For fiber fineness, $F_2/F_{2:3}$ stacked lines of pop. 07 (with *Gt*-allele in chromosome 07 and *Gm*-allele in chromosome 11) and pop. 11 (with *Gm*-alleles in chromosomes 11 and 25) showed 5-11% (**Figure 5.3h**) and 16-24% decrease (**Figure 5.3j**) in MIC compared to the parental controls. Similarly, stacked lines of pop. 11 (with *Gm*-alleles in chromosomes 11 and 25) showed 2-13% higher STR than the parental controls (**Figure 5.3l**). Also, besides tangible stacking effects in pop. 18 and pop. 19 (discussed earlier), stacked individuals in pop. 02, pop. 04, pop. 05, and pop. 07 also showed increased ELO of 15-26% (**Figure 5.3d**), 12-18% (**Figure 5.3g**), 3-12% (**Figure 5.3m**), and 7-17% (**Figure 5.3e**) over the parental controls.

For digenic models with interactions, five were significant at set thresholds ($p < 0.005$), while 19 nonredundant interactions were detected at marginal thresholds ($p < 0.05$) (**Supplemental Table S5.11**). In one such example, fuzz color locus interacted with *Gm*-alleles introgressed in chromosome 25 to influence UHM in two different populations [i.e., pop. 01 (2014) and pop. 04], while the same interaction in pop. 01 (2014) also influenced UI and SFC, thereby affecting the three genetically correlated traits (Wang et al. 2017a) in pop. 01 (2014).

Discussion

Although elite cultivars of Upland cotton have a narrow genetic base, introgressive breeding has introduced a preponderance of novel allelic variation into Upland cotton gene pool, a portion of which is quantitative in nature and has also been Mendelized through AB-QTL and backcross-self approaches. In

the current study, we developed and tested 21 early generation segregating populations (i.e., F_2 and $F_{2:3}$) to further assess QTL effects and 'stability' of eight fiber quality QTL introduced into *G. hirsutum* from *G. tomentosum* (Waghmare et al. 2005; Zhang et al. 2011; Waghmare et al. 2016) and *G. mustelinum* (Wang et al. 2016a; Wang et al. 2016b; Wang et al. 2017a; Wang et al. 2017b). Introgressed donor segments in backcross-selfed lines are in near-isogenic state and are expected to show little recombination between marker and QTL, thus detected phenotypic effects at SSR(s)-tagged sites accurately reflect effects of the QTL. To investigate effects of such QTL upon being transferred to other cultivated backgrounds (Paterson et al. 1990; Chee et al. 2005b), we targeted SSR(s)-tagged QTL regions employing MAS in early generation QTL-stacked populations. Several GA230- and R01-populations and selected $F_{2:3}$ lines showed favorable shifts in population mean for MIC, ELO, and STR, the three targeted traits in the current study (**Supplemental Tables S5.03 and S5.04**). To our knowledge, the current study is the first that brings together chromatin segments from four different species (i.e., *G. hirsutum*, *G. tomentosum*, *G. barbadense* and *G. mustelinum*). Our exploratory results provide a cautiously optimistic overview of QTL-pyramiding using interspecific alleles as one avenue toward a new 'adaptive peak' of cotton fiber quality.

QTL showing stable expression of genetic effects are amenable to molecular breeding. Several promising research and breeding endeavors have built upon resources that came out of early QTL analyses. For example, a major QTL conditioning high fiber strength was repeatedly detected in populations involving S-7235 as a source and TM-1, an Upland cotton line, as a recipient parent (Yuan et al. 2001; Guo et al. 2003; Zhang et al. 2003; Shen et al. 2005; Shen et al. 2007; Chen et al. 2009). The QTL was also detected and tagged with dozens of SSR markers in early generation segregants involving two additional backgrounds (Kumar et al. 2012), suggesting that this QTL can be reliably used to improve fiber strength in Upland cotton by the QTL-MAS approach. In the current study, we detected significant effects for several fiber quality QTL when transferred to additional *G. hirsutum* backgrounds beyond their near isogenic hosts.

G. mustelinum introgression in chromosome 11 spanning SSR loci MUSS123 and NAU3377 was associated with increased fiber elongation (qELO-11-1 or EL11.1) in BC_3F_2 (R^2=30.7%), $BC_3F_{2:3}$ (R^2=21.6%), and in combined analysis (BC_3F_2, BC_3F_2:$_3$ and $BC_3F_{2:4}$) where its additive effect explained 11.6% of total phenotypic variance for ELO (**Supplemental Table S5.01;** Wang et al. 2016a; Wang et al. 2016b). Selected individual B16-78 showed an average ELO of 7.0% in first two backcross-selfed generations and was homozygous for *Gm*-introgression at diagnostic SSR locus MUSS123. In the current study, qELO-11-1 was detected in three out of four populations in which it segregated (one in GA230 and two in R01-populations; two in 2013 and one in 2014) and contributed significantly to phenotypic variances in ELO in each (r^2 = 28-29%), suggesting that it is a major 'stable' QTL that transcends genetic backgrounds, an assertion further supported by the QTL's significant genotype effect (G) among GA230-selections. However, significant main-effect was not detected in pop. 07, which also segregated at MUSS123 and NAU3377, but showed monomorphic profile for NAU3341. Since interval mapping placed QTL position between MUSS123 and NAU3341 in three other populations (i.e., pop. 02, pop. 09 and pop. 10), it is possible that qELO-11-1 was lost due to recombination in the parental F_1 selection that was selfed to develop pop. 07. Nevertheless, mean ELO of pop. 07 and several selected lines were significantly higher than parental controls, while a marginally significant ($p < 0.05$) digenic interaction involved *Gm*-allele at chromosome 11 locus partially explained higher ELO in pop. 07. Another plausible explanation could be transgressive segregation between the backgrounds, masking the actual QTL effect at the target locus. As such, transgressive segregation could be due to epistatic effects of favorably interacting alleles. We developed and tested parental crosses (i.e. GA230 or R01 with PD94042) to isolate actual QTL effects from G x B interactions. Positive transgression for fiber elongation in parental crosses was not adequate to explain the significant shift in ELO values of pop. 07, warranting further investigation.

qELO-1-1 was detected in POP15 in two backcross-selfed generations [BC_3F_2 (R^2=14.5%) and $BC_3F_{2:3}$ (R^2=9.9%)] and in combined analysis (BC_3F_2, BC_3F_2:$_3$ and $BC_3F_{2:4}$) where its additive effect explained 3.71% of total phenotypic variance for ELO. Selected individual B15-70 showed an average ELO

of 6.9% across generations (Wang et al. 2016a) and was homozygous for *Gm*-introgression at diagnostic SSR locus NAU2095. In the current study, qELO-1-1 effect was detected in four out of seven populations in which it segregated (two in R01 and two in GA230 background; one in 2013 and three in 2014) and contributed 5-12% of total phenotypic variances in ELO in each, suggesting that it is a 'minor effect' QTL. A suggestive digenic interaction ($p < 0.05$) involving NAU2095 was associated with ELO in pop. 12, while the locus displayed significant among-family genotype effect ($p < 0.0005$) and G x F interaction in GA230 populations ($p < 0.0048$). Although main effects of qELO-1-1 were not significant in three populations (pop. 12, pop. 19, and pop. 20), F_2 mean and average elongation of selected $F_2/F_{2:3}$ lines of pop. 19 and two pop. 20 selections (viz. 244-166 and 244-176) were significantly higher than control means. In other words, the contrasts between NAU2095 genotypic classes were not statistically different for these populations, but several selections carrying *Gm*-alleles at NAU2095 had remarkably high elongation values. In pooled analysis of $F_2/F_{2:3}$ selections of pop. 18, lines carrying homozygous NAU2095 *Gm*-alleles averaged 17% greater fiber elongation then those with homozygous *Gh*-alleles (**Figure 5.3b**). Therefore, qELO-1-1 is a valuable candidate for fine mapping and QTL pyramiding for increasing fiber elongation in *G. hirsutum* cultivars.

Another QTL target involved *G. mustelinum* introgression on chromosome 25 between marker loci STS511 and BNL4001b, which was associated with increased fiber strength (qSTR-25-1) in BC_3F_2 (R^2~14%), $BC_3F_{2:3}$ (R^2=17.2%), and $BC_3F_{2:4}$ (R^2=13.2%) (Wang et al. 2017b). Selected parent B17-008 showed STR of 35.4 g/tex in $BC_3F_{2:3}$ and was homozygous for *Gm*-introgression at diagnostic SSR loci BNL3264 and BNL4001b. In the current study, marker-trait association was significant for higher strength in one R01-population (pop. 13; mean = 32.28±2.27 g/tex), further supported with higher strength (mean = 33.54±0.70 g/tex) of its selectively advanced $F_{2:3}$ lines. Compared to the parental line with highest strength (R01; mean = 30.42±2.04), 81%, 68%, 43%, 37%, 27%, 13% and 2% of F_2 individuals from pop. 13, pop. 01 (2014), pop. 01 (2013), pop. 11, pop. 06, pop. 15, and pop. 04 had higher strengths, respectively (data

not shown), which indicates that the extent of positive transgression was not uniform among populations that inherited qSTR-25-1.

In the original study (Wang et al. 2017b), overdominance at the BNL4001b locus was also associated with increase or decrease in MIC in BC_3F_2 and $BC_3F_{2:3}$ generation, respectively, suggesting presence of genes conditioning different effects contributed by *G. mustelinum* (Wang et al. 2017b). In the current study, average MIC values were significantly lower in five out of six F_2 populations segregating at the introgressed site, while marker-trait association was only detected in pop. 04. Further, we were able to track *Gm*-introgression on chromosome 11 in a region corresponding to SSR marker NAU1014 and a phenotypic marker (green fuzz color) carried by NIIL B17-008. Favorable QTL effects from *G. mustelinum* introgression were consistently detected in two different backgrounds and generations, making the region attractive for fine mapping and QTL-MAS. However, this QTL was not detected in the original study, suggesting that we detected a novel effect in new backgrounds associated with an introgressed *Gm*-allele.

In the current study, several *Gt*- and one *Gm*-QTL (i.e., qELO-21-1) effect(s) were either not detected or were not 'stable' across populations and/or generations. Specifically, *Gt*-QTL effects were elusive in F_2 mapping populations. Of three *Gt*-QTL for fiber elongation (i.e., qFE11.1, qFE14.1, and qFE21.1), targeted effects were detected in none (for qFE21.1) or in one (for qFE11.1 and qFE14.1) population. In the original *Gt*-QTL study, introgressed allele(s) from *G. tomentosum* contributed relatively small proportions of overall phenotypic variation for fiber quality traits (Zhang et al. 2011). For example, *Gt*-QTL targeted in the current study explained 4.5-9.0% variation in fiber phenotype recorded at Lubbock, Texas. Specifically, qFE14.1 and qFE21.1 explained significant variation ($p < 0.0001$) in BC_3F_2 (R^2=7.8-9%) and BC_3F_3 (R^2=4.5-5.7%), while qFE11.1 and qFF07.1 explained significant variation in BC_3F_2 (R^2=6.7%) and BC_3F_3 (R^2=6%) generations, respectively, with each QTL reaching significance only at Lubbock. Further, with respect to backcross-selfed BC_3F_3 lines grown at two different locations (Lubbock, TX and Tifton, GA) in 2003, distribution of fiber elongation at Lubbock (mean±SD = 5.69±0.86%) was

broader compared to Tifton (mean±SD = 8.36±0.44%), indicating that narrow variation precluded detection of small effect *Gt*-QTL at Tifton (Zhang et al. 2011). It is also noteworthy that in prior study, genotype contrasts were inferred from a small number of individuals retaining introgressed segments (at heterozygous state) in advanced segregating populations and fiber elongation showed significant and pronounced environmental influence (Zhang et al. 2011; Waghmare et al. 2016). In the current study, each target *Gt*-QTL was represented in several segregating populations and perhaps because we targeted selectively retained *Gt*-introgressions, segregation distortions were not observed during population development. However, the current study involved QTL-stacks between *Gt*- and *Gm*-QTL (in *G. hirsutum* genetic backgrounds), in which major effects of *Gm*-QTL or *Gh*-QTL could have overshadowed the effects of minor *Gt*-QTL by increasing the total phenotypic variation, where minor QTL effects are basically too small to be detected (Zhang et al. 2003). Therefore, a paucity of detectable phenotypic effects associated with *Gt*-QTL could be due to substantial environmental influence, background (i.e., epistatic) effects, and/or lack of additivity (i.e., mode of action).

Although the major portion of genetic variance in fiber quality traits has been attributed to additive gene action (Wang et al. 2007; Ng et al. 2014a), several studies have underscored the importance of epistasis as a genetic basis of heterotic responses in cotton. For example, significant epistatic interactions have been reported for cotton morphological and phenological traits (Guo et al. 2008; Song and Zhang 2009; Liang et al. 2014; Liu et al. 2014), yield and yield components (Jia et al. 2014), insect-pest and disease resistance (Ulloa et al. 2010; He et al. 2014), and fiber quality traits (He et al. 2008; Saha et al. 2011; Cao et al. 2014; Guo et al. 2014; Yuan et al. 2014; Song et al. 2017). In the current study, we detected five significant digenic interactions, with three interactions involving marker loci that did not show significant effects in single marker analysis. Similar observations were reported by He et al. (2008). Further, fiber quality QTL are significantly influenced by backgrounds in which these QTL are deployed, suggesting the importance of multilocus interactions (Chee et al. 2005a; Chee et al. 2005b; Draye et al. 2005; Zhang et al. 2011). Our study also detected six significant G x F interactions at three marker loci (**Supplemental Table S5.10**).

Together, these reports highlight a need to consider QTL by QTL interaction effects in MAS breeding, as such epistatic interactions could be one reason for inconsistency among marker-trait associations across environments, generations, genetic backgrounds, and mapping studies. Accordingly, QTL-stacked populations are desirable for testing epistatic interactions among fiber quality QTL. Further, individual fiber quality QTL often contribute relatively little to overall phenotypic variation (<10%) (Said et al. 2015b; Jamshed et al. 2016), which is further exacerbated by profound environmental influence (Paterson et al. 2003; Lacape et al. 2010), making QTL pyramiding attractive for achieving commercially important levels of improvement in fiber quality traits of Upland cotton.

QTL-MAS pyramids targeting fiber quality traits have been reported in Upland cotton, which mostly focus on fiber length (Souder et al. 2004; Dong et al. 2009) and fiber strength (Yi et al. 2004; Guo et al. 2005; Yuan et al. 2014). For example, Yuan et al. (2014) pyramided two fiber strength QTL and showed that stacked lines had higher fiber strength than lines carrying single QTL. Similarly, Cao et al. (2014) developed improved QTL-stacked lines carrying *G. barbadense* introgression associated with increased fiber length, strength, and micronaire. Guo et al. (2014) also detected and performed MAS of three QTL (one each for fiber length, strength, and micronaire) where favorable alleles were contributed by *G. barbadense*. In the current study, we also detected enhanced fiber properties associated with several QTL-stacks. For example, selectively advanced QTL-stacked (doubly homozygous) $F_2/F_{2:3}$ lines showed significantly reduced micronaire (i.e., improved fineness), increased elongation, and higher strength than singly homozygous lines and parental checks, which supports the merit of QTL-MAS pyramiding for enhanced fiber attributes in Upland cotton.

Co-localization of QTL underlying different fiber quality traits partially explains moderate to strong correlation among fiber phenotypes (Zhang et al. 2011). Pleiotropic or close linkage relationships among fiber quality QTL were also reported in *G. tomentosum* (HT) and *G. mustelinum* (HM). For example, an ELO QTL (qFE14.1) and a UI (qFU14.1) QTL co-located in HT populations, where favorable allele for

ELO and UI came from *G. tomentosum*. qFF14.1 and qFU14.1 showed significant G x B interactions, and both were detected in *G. hirsutum* x *G. barbadense* F_2 populations (Paterson et al. 2003). We also detected a significant main-effect of qFU14.1 in pop. 02 and suggestive main-effects of qFE14.1 and qFF14.1 in pop. 01 (2014), while contrasts between $F_2/F_{2:3}$ selections (pop. 02) homozygous for *Gh*- and *Gt*-alleles at the locus was also significant for ELO. Similarly, in *G. hirsutum* x *G. mustelinum* NIILs, fiber strength QTL (qSTR-25-1) co-located with qMIC-25-1 and qUHM-25-1, which was also evident in our current study and corresponds to evidence that common genetic loci may affect both traits (Chee et al. 2005b).

Correlations among fiber quality traits were generally in line with prior reports (Lacape et al. 2005; Shen et al. 2007). For example, *Gm*-alleles of marker NAU2095 associated with fiber elongation in two GA230- and two R01-populations, were also associated with unfavorable fiber quality traits that are characteristically correlated with fiber elongation. Specifically, positive correlation between MIC and ELO in F_2 generation is partially explained by additive effects of *Gm*-alleles at NAU2095 locus in pop. 08 and pop. 18 and with a decrease in STR in pop. 03 and pop. 08. Although contrasts among genotypic classes were not statistically significant, concurrent increase in ELO and MIC and a decrease in STR was also evident in pooled analysis of selected $F_2/F_{2:3}$ lines of pop. 18, pop. 19, and pop. 20, the three populations segregating at NAU2095 locus.

Similarly, negative correlation between STR and ELO is partially explained by the effect at *Gm*-introgression in chromosome 11 (corresponding to marker loci MUSS123, NAU3341 and NAU3377), where *Gm*-alleles increase ELO and decrease STR in pop. 02 (p<0.001 and R^2=9-15%) and pop. 09. The concurrent decrease in fiber strength associated with *Gm*-alleles was also evident in the combined analysis. The results are consistent with Wang et al. (2017b), where *G. mustelinum* introgression in chromosome 11 was also associated with decreased fiber strength (qSTR-11-1) in BC_3F_2 (R^2=11.9%) and $BC_3F_{2:3}$ (R^2=29.2%). However, two notable exceptions were observed where STR and ELO were positively correlated [pop. 07 (r=0.27, p < 0.01) and pop. 16 (r=0.33, p < 0.01)] and these exceptions could be due to

differential transmission of regulatory genes spanning QTL 'hotspots', where different co-segregating haplotypes affect phenotypes differentially (Paterson et al. 2012).

In the current study, a number of crosses with *G. hirsutum* x *G. mustelinum* BC_3F_3 NIILs showed atypical plant characteristics (miniature stature, smaller bolls, larger seeds, late maturity), indicating substantial linkage drag associated with introgressed *Gm*-alleles. Many populations were late in flowering and maturity, consequently producing insufficient fiber for HVI analysis. Upon replanting in 2014, a few late maturing populations did not fare better, suggesting that exotic introgressions carried maturity determinants. Of interest, crosses involving NIIL B17-008 showed several distinct phenotypes including large seeds with green fuzz color. Average fiber fineness of secondary segregating populations involving B17-008 (pop. 01, pop. 04, pop. 11, pop. 13, and pop. 15) and their selectively advanced $F_{2:3}$ lines were significantly lower than parental controls. Further, pop. 4 and pop. 15 showed significantly high short fiber content (%) both in F_2 and their $F_2/F_{2:3}$ selections. Nevertheless, several F_2 individuals from pop. 13 and pop. 15 and average phenotypes of lines 242-004 (3.68 MIC and 32.81 g/tex) and 241-036 (3.83 MIC and 34.35 gf/tex) selected from the respective populations combined increased fiber fineness (i.e., reduced MIC) with higher strength, indicating that these traits can be improved simultaneously.

The heritability of fiber quality characteristics is moderate to high (Meredith and Bridge 1984; May 1999), indicating that these attributes can be manipulated in early segregating generations. In pooled analysis, we found that genotype contribution to phenotypic variations were larger for MIC (51-56%), ELO (58-68%), and UML (51-60%) than for UI (~39%) and SFC% (32-35%). Also, G x E contributions were not significant at set thresholds ($p < 0.0001$). As such, we were able to validate main-effects of several target QTL in early segregating generations. While construction of near-isogenic lines to fine-map *Gm*-QTL regions are underway (Wang et al. 2016a; Wang et al. 2017a; Wang et al. 2017b), estimation of yield potential in currently employed backgrounds needs to be done. Since there are precedents of fiber quality QTL expressing erratically across different environments, generations, and/or genetic backgrounds

(Paterson et al. 2003; Sun et al. 2012; Fang 2015; Jamshed et al. 2016; Liu et al. 2017), we are currently investigating *Gt*-QTL-stacked populations (i.e., *Gt*-QTL x *Gt*-QTL) in four to six different *G. hirsutum* backgrounds representing four major cotton production regions of the United States. Besides validation of QTL effects, these populations would allow *G. mustelinum* alleles to be more readily accessible to regional breeding programs.

Conclusion

The present study demonstrates the practical use of QTL-MAS strategy for transferring fiber quality QTL to *G. hirsutum* cultivars and validating their effects in early generation segregants. Our results indicate that effectiveness of QTL stacking depends upon several factors including size of QTL effect (i.e., major or minor), G x E interactions, background effects (i.e., epistasis), population characteristics (i.e., size and nature), and MAS strategy. The development and characterization of several phenotypically superior fiber quality lines in partial genetic backgrounds of *G. hirsutum* cultivars and validated QTL and QTL-stacks represent a valuable resource for marker-assisted breeding for enhanced fiber quality of commercial cultivars.

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Table 5.1 Distribution of average fiber quality phenotypes of parental checks and QTL-stacked populations in partial genetic backgrounds of *G. hirsutum*.

Plant materials	Epithet	Year	Background	Specifics[a]	Size[b]	Fiber quality phenotypes[c]					
						Micronaire (MIC)	Upper Half Mean Length UHM (inch)	Length Uniformity Index UI (%)	Fiber Strength STR (gm/tex)	Fiber Elongation ELO (%)	Short Fiber Content SFC (%)
Parental checks											
CA3093	CA93 (2013)	2013		Check; recurrent parent of *Gt*-QTL introgressions	8	4.86±0.34	1.02±0.03	83.19±1.18	27.05±1.24	6.14±0.31	7.45±0.61
CA3093	CA93 (2014)	2014			5	5.08±0.43	1.04±0.06	82.85±1.06	27.90±1.01	6.15±0.39	7.85±0.63
GA2004230	GA30 (2013)	2013		Parental check	18	4.57±0.25	1.15±0.04	84.33±1.05	29.01±1.30	5.54±0.33	7.21±0.43
GA2004230	GA30 (2014)	2014			9	4.75±0.23	1.17±0.05	83.89±0.97	29.43±1.04	5.76±0.53	7.65±0.23
R01-40-08	R01 (2013)	2013		Parental check	16	4.34±0.57	1.18±0.04	84.89±0.87	30.42±2.04	6.25±0.52	7.03±0.42
R01-40-08	R01 (2014)	2014			10	4.70±0.37	1.15±0.04	83.69±1.45	29.83±1.44	5.83±0.79	7.74±0.79
PD94042	PD42 (2013)	2013		Check; recurrent parent of *Gm*-QTL introgressions	14	4.74±0.25	1.10±0.04	84.21±0.81	29.70±1.68	5.51±0.24	7.20±0.42
PD94042	PD42 (2014)	2014			5	5.21±0.18	1.06±0.02	82.65±0.64	27.88±1.04	6.10±0.35	7.85±0.63
GA2004230 x PD94042	GA30xPD42	2013		Check; F_2 population	75	4.24±0.40	1.12±0.06	83.40±1.52	28.61±1.98	5.72±0.62	7.61±0.74
R01-40-08 x PD94042	R01xPD42	2013		Check; F_2 population	80	4.55±0.52	1.15±0.06	84.95±1.25	29.73±2.20	6.58±0.78	6.97±0.51
QTL-stacked populations											
Pop. 01	Pop. 01 (2013)	2013	R01	*Gt(14)xGm(25)xGb(01)*	46	3.84±0.47	1.06±0.05	82.65±1.72	30.26±2.77	6.94±0.76	7.73±0.91
Pop. 01	Pop. 01 (2014)	2014	R01	*Gt(14)xGm(25)xGb(01)*	65	4.19±0.59	1.20±0.05	85.18±1.24	31.46±2.44	5.77±0.67	7.18±0.49
Pop. 02		2013	R01	*Gt(14)xGm(11/21)xGb(01)*	132	5.02±0.44	1.01±0.06	82.91±1.33	28.16±2.17	6.80±0.89	7.08±0.82
Pop. 03	Pop. 03 (2013)	2013	R01	*Gt(14)xGm(01)xGb(01)*	64	4.35±0.48	1.17±0.05	84.62±1.07	28.09±1.62	7.10±0.82	7.23±0.51
Pop. 03	Pop. 03 (2014)	2014	R01	*Gt(14)xGm(01)xGb(01)*	58	4.90±0.45	1.15±0.06	84.42±1.07	28.18±1.46	7.26±0.63	7.22±0.56
Pop. 04		2013	R01	*Gt(07)xGm(25)x1*	94	4.06±0.57	1.07±0.07	82.56±1.19	26.23±2.01	6.42±0.73	7.84±0.71
Pop. 05		2013	GA30	*Gt(14)xGm(21)*	62	4.84±0.40	1.03±0.04	83.81±1.05	27.22±1.61	5.80±0.56	7.27±0.86
Pop. 06		2014	GA30	*Gt(21)xGm(25)*	33	4.71±0.58	1.08±0.07	83.03±1.19	29.20±1.83	5.20±0.62	8.08±0.76
Pop. 07		2013	GA30	*Gt(07)xGm(11/21)*	105	4.28±0.44	1.06±0.05	83.54±1.04	26.22±1.58	6.43±0.65	7.32±0.69
Pop. 08		2014	GA30	*Gt(14)xGm(01)*	47	5.61±0.74	1.06±0.09	83.29±1.39	27.17±2.68	6.49±0.70	7.80±0.85
Pop. 09		2014	R01	*Gt(07)xGm(11)xGb(01)*	34	5.42±0.70	0.98±0.09	82.16±2.03	26.90±2.24	7.18±1.89	8.48±2.58
Pop. 10		2013	GA30	*Gt(21)xGm(11)*	57	4.26±0.43	1.06±0.06	83.36±1.23	25.95±1.57	6.08±0.79	7.59±0.78
Pop. 11		2013	R01	*Gt(21)xGm(25)*	75	4.06±0.45	1.14±0.07	84.34±1.59	29.93±2.12	5.14±0.69	7.17±0.62
Pop. 12		2013	R01	*Gt(21)xGm(01)*	47	4.48±0.34	1.09±0.06	83.63±1.45	27.89±1.62	6.21±0.55	7.41±0.77
Pop. 13		2013	R01	*Gt(11)xGm(25)*	62	3.84±0.56	1.14±0.06	85.18±1.39	32.28±2.27	6.00±0.67	7.07±0.68
Pop. 14		2013	R01	*Gt(11)xGm(21)*	33	3.89±0.52	1.04±0.06	83.64±1.31	27.28±2.11	4.75±0.63	7.20±0.71
Pop. 15		2013	GA30	*Gt(11)xGm(25)*	48	3.31±0.54	1.11±0.05	82.75±1.57	28.58±2.29	4.96±0.58	8.24±0.87
Pop. 16		2013	GA30	*Gt(11)xGm(21)*	69	4.72±0.47	1.07±0.05	83.4±10.34	27.54±2.24	4.80±0.70	7.72±0.97
Pop. 17		2014	R01	*Gt(07)xGm(21)*	36	4.74±0.44	1.15±0.05	84.52±1.20	29.84±2.09	5.65±0.75	7.36±0.64
Pop. 18		2013	R01	*Gt(07)xGm(01)*	96	5.41±0.48	1.01±0.06	83.09±1.30	27.98±2.02	7.27±0.72	7.52±0.85
Pop. 19		2013	GA30	*Gt(07)xGm(01)*	76	4.99±0.49	0.99±0.05	82.48±1.24	25.47±1.53	7.45±0.79	7.96±1.18
Pop. 20		2013	R01	*Gt(11)xGm(01)*	60	4.60±0.65	1.03±0.06	83.86±1.15	28.07±2.29	6.33±0.62	7.34±0.85
Pop. 21		2014	GA30	*Gt(11)xGm(01)*	110	4.45±0.68	1.07±0.05	83.12±1.31	26.28±1.58	6.69±0.75	7.82±0.86

[a] CA3093 and PD94042 are recurrent *G. hirsutum* backgrounds of advanced-backcross near isogenic introgression lines (NIILs) carrying *G. tomentosum* (Zhang et al. 2011) and *G. mustelinum* (Wang et al. 2016) QTL. *Gt(07)*, *Gt(11)*, *Gt(14)*, and *Gt(21)* refer to *G. tomentosum* introgressions in chromsomes 7, 11, 14, and 21, respectively. *Gm(01)*, *Gm(11)*, *Gm(21)*, and *Gm(25)* refer to G. *mustelinum* introgressions in chromosomes 1, 11, 21, and 25, respectively. Gb(01) refers to *G. barbadense* introgression in chromosome 1. [b] Size references to *G. hirsutum* parental checks denote number of plots, while those for F_2 populations refer to number of individuals in the population. [c] HVI phenotypes.

Table 5.2 Marker-trait associations for fiber fineness, elongation, and strength in QTL-stacked F_2 populations.

Trait	Population	Background	Locus/interaction	Source	Effect			Test of Significance P > F		R^2
					a	d	d/a	Marker	Model	
Micronaire (MIC)	Pop. 01 (2013)	R01	Fuzz color	*Gm*	-0.54	-	-	<.001		0.23
	Pop. 01 (2014)	R01	NAU1014d	*Gh/Gm*	0.27	0.02	-0.08	0.018	0.009	0.19
			NAU5027c	*Gh/Gt*	0.13	-0.35	2.76	0.040		
	Pop. 04 (2013)	R01	NAU2432c	*Gh/Gt*	-0.16	0.00	-0.03	0.034	<.001	0.16
			NAU2238a§	*Gm*	-0.31	-	-	0.004		
			Fuzz color	*Gm*	-0.26	-	-	0.006		
	Pop. 06 (2014)	GA30	NAU3053c	*Gh/Gh*	0.59	0.14	-0.24	0.004		0.26
	Pop. 08 (2014)	GA30	NAU2095c	*Gh/Gm*	0.47	0.33	-0.69	0.001		0.25
	Pop. 11 (2013)	R01	NAU3074d	*Gh/Gt*	0.14	0.19	-1.41	0.015	0.004	0.15
			Fuzz color	*Gm*	-0.31	-	-	0.013		
	Pop. 12 (2013)	R01	NAU3731c§	*Gh/Gt*	-0.25	0.04	0.15	0.001		0.27
	Pop. 15 (2013)	GA30	NAU1014d	*Gt/Gm*	0.31	0.05	-0.17	0.007	<.001	0.28
			Fuzz color	*Gm*	-0.58	-	-	0.002		
	Pop. 18 (2013)	R01	NAU2095c†	*Gh/Gm*	0.19	0.00	0.01	0.006		0.12
	Pop. 19 (2013)	GA30	NAU2432c†	*Gh/Gt*‡	-0.20	-0.04	-0.19	0.013		0.14
	Pop. 21 (2014)	GA30	NAU1014d	*Gh/Gt*	-0.24	0.30	1.25	0.003		0.10
Fiber Strength (STR)	Pop. 02 (2013)	R01	MUSS123c	*Gh/Gm*	1.07	-0.13	-0.12	0.001		0.09
			NAU3377c	*Gh/Gm*	1.43	0.22	0.15	<.001		0.15
			NAU3341c	*Gh/Gm*	1.08	0.00	0.00	<.001		0.10
	Pop. 03 (2014)	R01	NAU5027c	*Gh/Gt*	0.84	0.14	0.17	0.003	<.001	0.25
			NAU2095c	*Gh/Gm*	0.77	-0.05	-0.06	0.004		
	Pop. 06 (2014)	GA30	Fuzz color	*Gm*	2.35	-	-	0.006		0.19
	Pop. 08 (2014)	GA30	NAU2095c	*Gh/Gm*	1.59	-1.36	-0.85	0.002		0.24
	Pop. 09 (2014)	R01	NAU3053c	*Gh/Gh*	0.79	-2.33	-2.96	0.028	0.004	0.32
			NAU3341c	*Gh/Gm*	0.27	2.13	8.00	0.019		
	Pop. 13 (2013)	R01	NAU2565	*Gm*	-1.92	-	-	<.001		0.16
			NAU2679	*Gm*	-1.92	-	-	<.001		0.16
Fiber Elongation (ELO)	Pop. 01 (2014)	R01	NAU1014d	*Gh/Gm*	0.34	-0.07	-0.22	0.028	0.008	0.20
			NAU5027c	*Gh/Gt*	-0.25	-0.23	0.90	0.030		
	Pop. 02 (2013)	R01	MUSS123c	*Gh/Gm*	-0.60	0.08	-0.13	<.001		0.19
			NAU3377c	*Gh/Gm*	-0.67	0.04	-0.06	<.001		0.20
			NAU3341c	*Gh/Gm*	-0.65	-0.12	0.18	<.001		0.25
	Pop. 03 (2013)	R01	NAU2095b	*Gm*	-0.47	-	-	0.050		0.12
	Pop. 08 (2014)	GA30	NAU2095c	*Gh/Gm*	-0.27	0.23	-0.85	0.038		0.14
	Pop. 09 (2014)	R01	NAU3341c	*Gh/Gm*	-1.36	-0.36	0.26	0.002		0.30
			NAU3377c†	*Gh/Gm*	-0.62	1.17	-1.89	0.036		0.23
			MUSS123c†	*Gh/Gm*	-0.98	0.43	-0.43	0.013		0.29
	Pop. 10 (2013)	GA30	NAU3341c	*Gh/Gm*	-0.54	0.10	-0.19	<.001		0.22
			MUSS123c†	*Gh/Gm*	-0.43	0.17	-0.39	0.005		0.21
	Pop. 12 (2013)	R01	NAU3731c†	*Gh/Gt*	0.33	-0.05	-0.15	0.005		0.32
	Pop. 18 (2013)	R01	NAU2095c	*Gh/Gm*	-0.29	-0.07	0.25	0.011	<.001	0.16
			NAU2432c	*Gh/Gt*	-0.29	-0.24	0.83	0.006		
	Pop. 21 (2014)	GA30	NAU1014d	*Gh/Gt*	0.15	0.38	2.52	0.019	0.008	0.11
			NAU2095c	*Gh/Gm*	-0.28	0.02	-0.08	0.051		

† significance reported for contrast between homozygous lines; ‡ Gh allele contributed by PD94042

§ model is also significant when replaced with other diagnostic SSR mapped to the region

Table 5.3 QTL for fiber related-traits in *G. hirsutum* populations introgressed with *G. mustelinum*

QTL	Population	Flanking markers	Additive[a]	d	d/a	LOD[b]	PVE (%)[c]
Fiber strength (gf/tex)							
qSTR-11-1	Pop. 02	MUSS123c-NAU3341c	1.51	-0.05	-0.03	4.93 (2.50)	0.20
	Pop. 09		0.26	2.12	8.15	1.90 (1.84)	0.03
Fiber elongation (%)							
qELO-11-1	Pop. 02	MUSS123c-NAU3341c	-0.78	-0.09	0.12	9.81 (2.50)	0.29
	Pop. 09		-1.36	-0.36	0.26	3.07 (1.75)	0.26
	Pop. 10		-0.54	0.09	-0.17	3.49 (1.84)	0.28

[a]a negative sign (-) of the additive effect indicates that the allele originated from *G. mustelinum* increases the value of the trait.

[b]values inside parenthesis are thresholds obtained from 1,000 permutations

[c]PVE, Phenotypic variance explained

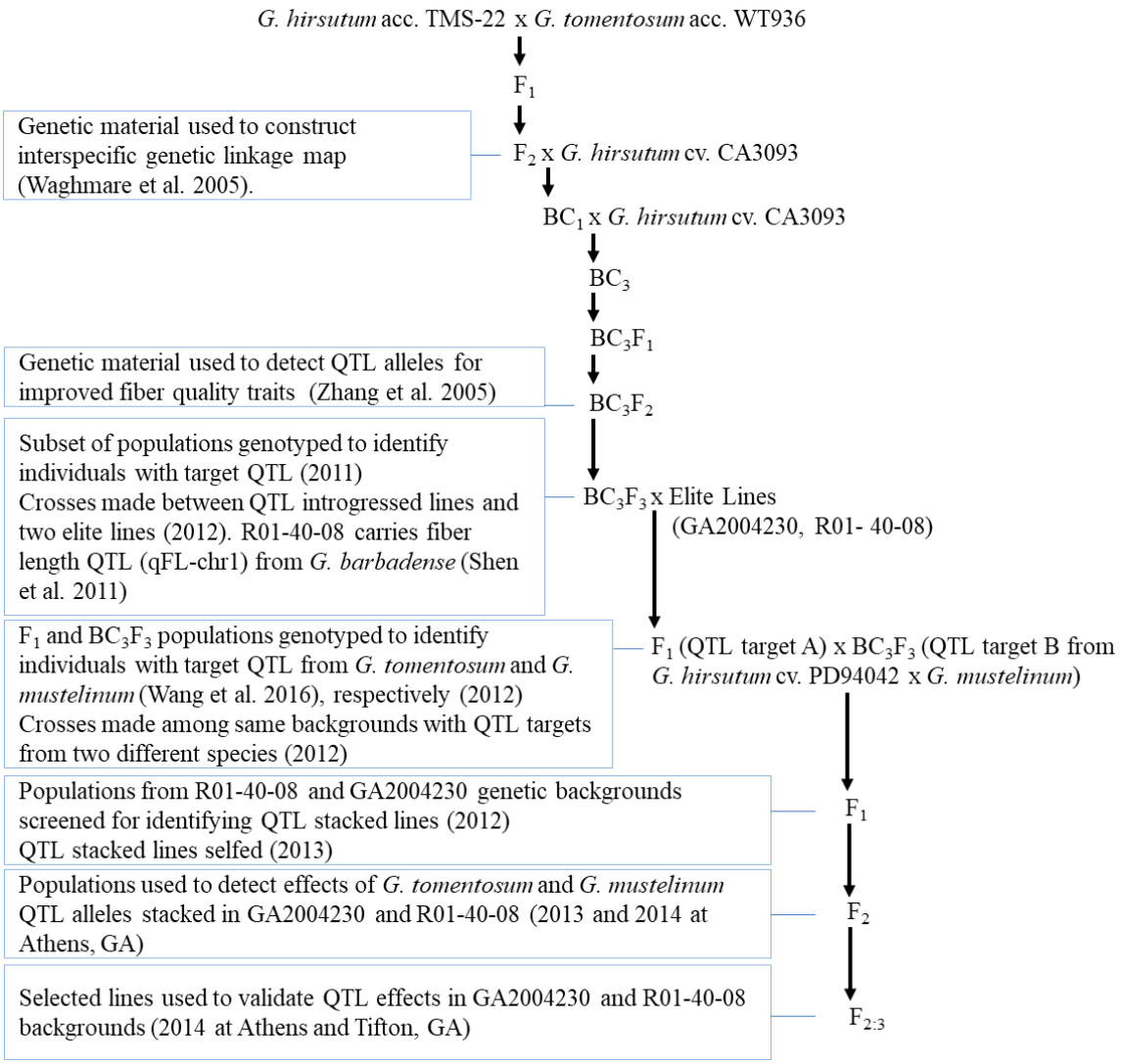


Figure 5.1 Development of populations with *Gossypium tomentosum*, *G. mustelinum* and *G. barbadense* QTL alleles stacked in *G. hirsutum* backgrounds.

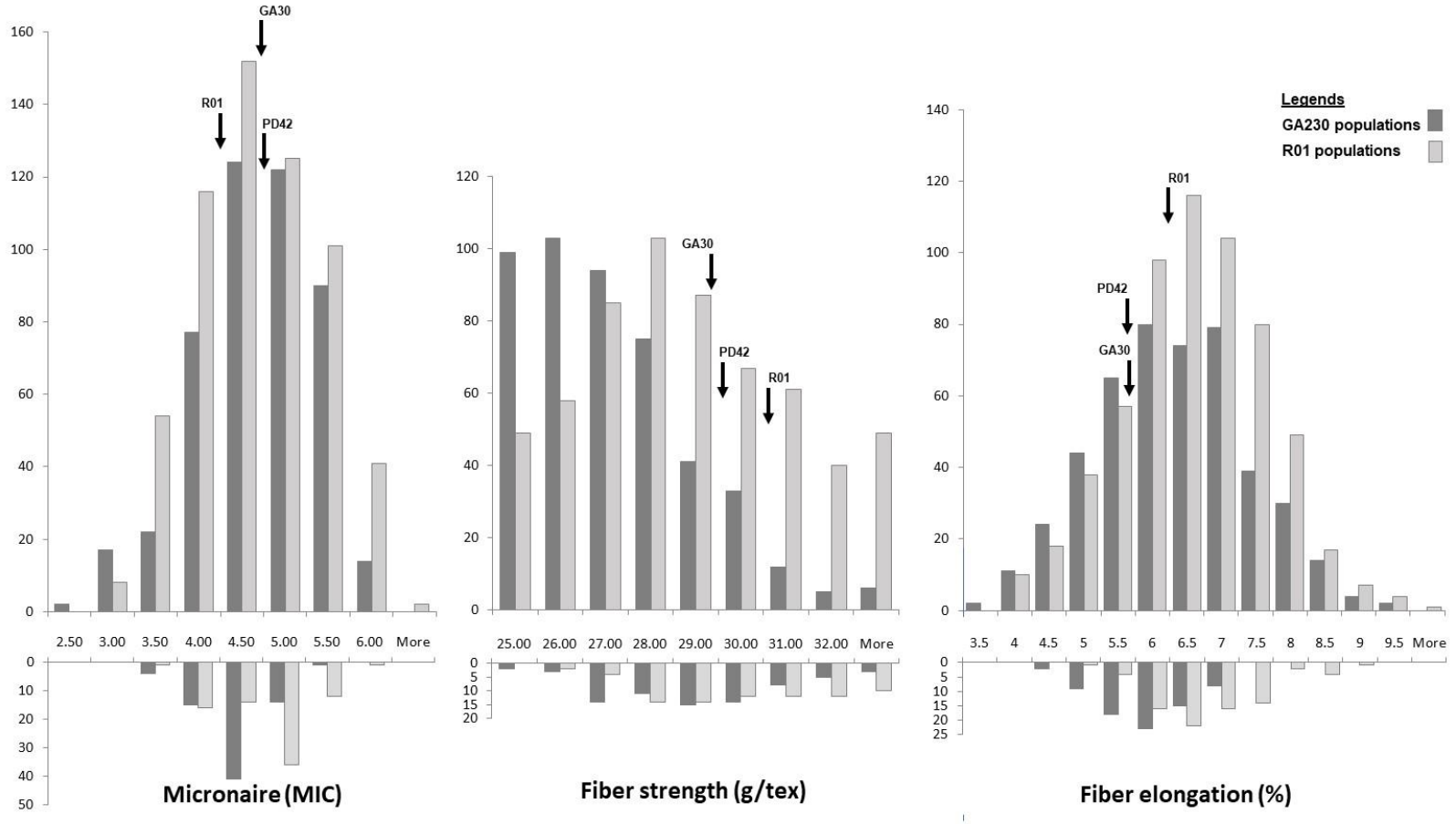
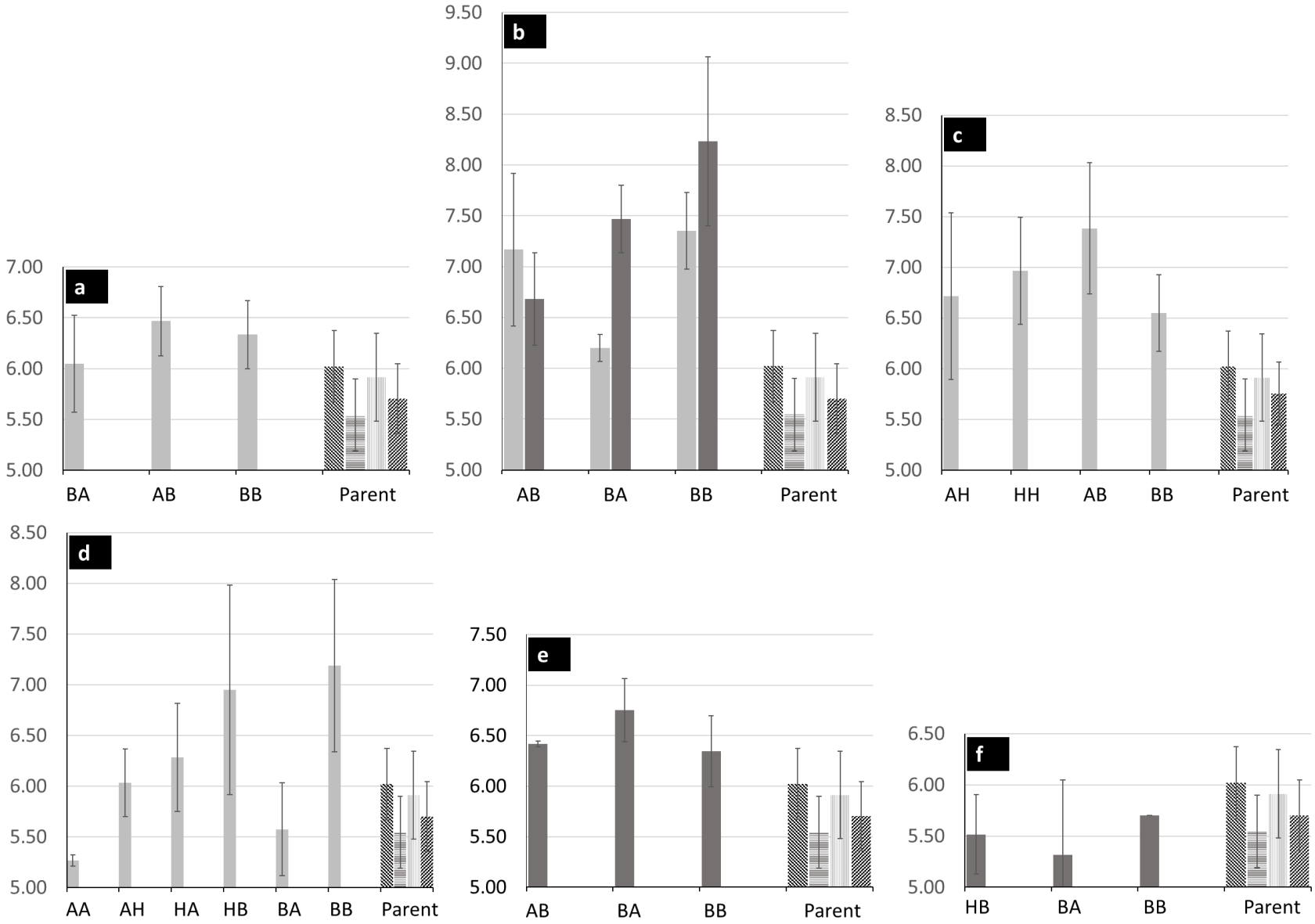


Figure 5.2 Distribution of micronaire, fiber strength, and fiber elongation phenotypes in F_2 populations carrying exotic alleles in *G. hirsutum* genetic backgrounds. Distribution of QTL-stacked F_2 populations shown above x-axis, while corresponding distribution in control crosses (i.e., GA200230 x PD94042 and R01-40-08 x PD94042) juxtaposed below abscissa as inverted bar-charts.

Figure 5.3 Mean trait values of digenic genotypes in pooled analysis of selectively advanced $F_2/F_{2:3}$ lines from QTL-stacked populations. Four *left bars* in each figure correspond to CA3093, GA2004230, R01-40-08, and PD94042. Figure 5.3a-5.3f and 5.3m shows average fiber elongation of three, three, four, six, three, three, and three digenic genotypes of selected lines from pop. 12 (a. NAU3731-NAU2095), pop. 18 and pop. 19 (b. NAU2432-NAU2095), pop. 20 (c. NAU1014-NAU2095), pop. 02 (d. NAU5027-NAU3341), pop. 07 (e. NAU2432-MUSS123), pop. 10 (f. NAU3074-NAU3377), and pop. 05 (NAU5027c-NAU3074d), respectively. 'A' refers to homozygous *Gh*-alleles at a locus, 'B' refers to either homozygous *Gt*-alleles (first character) or homozygous *Gm*-alleles (second character), and 'H' refers to heterozygous. Figure 5.3g-5.3k shows average fiber fineness (also fiber elongation in 5.3g) of three, three, five, six, and nine digenic genotypes of selected lines from pop. 04 (g. NAU2432-NAU2565), pop. 07 (h. NAU2432-MUSS123), pop. 18 and 19 (i. NAU2432-NAU2095), pop. 4, pop. 11, pop. 13 and pop. 15 (j. NAU1014-NAU2565), and pop. 04, pop. 11, pop. 13 and pop. 15 (k. NAU1014-Fuzz color), respectively. For Figure 5.3g-5.3i, 'A' refers to homozygous *Gh*-alleles at a locus and 'B' refers to either homozygous *Gt*-alleles (first character) or homozygous *Gm*-alleles (second character). For figure 5.3j-5.3k, 'A' refers to homozygous *Gh*-alleles (first character) for pop. 04 and pop. 11, but refers to homozygous *Gt*-alleles (first character) for pop. 13 and pop. 15 since these populations were segregating for *Gt*- by *Gm*-alleles at NAU1014 locus. Accordingly, 'B' refers to homozygous *Gm*-alleles. For markers NAU2565, 'C' refers to presence of a dominant *Gm*-allele at NAU2565 locus, while 'N' refers to homozygous null alleles. Similarly, fuzz color G, M, and W correspond to *green*, *white*, and *mixed* phenotypes, respectively. Figure 5.3l shows average fiber strengths of six digenic genotypes of selected lines from pop. 04, pop. 11, pop. 13, and pop. 15. 'N' corresponds to null allele, while 'C' corresponds to presence of *Gm*-allele at NAU2565 locus. Fuzz color G, M, and W correspond to *green*, *white*, and *mixed* phenotypes, respectively. Error bars show standard deviations around means.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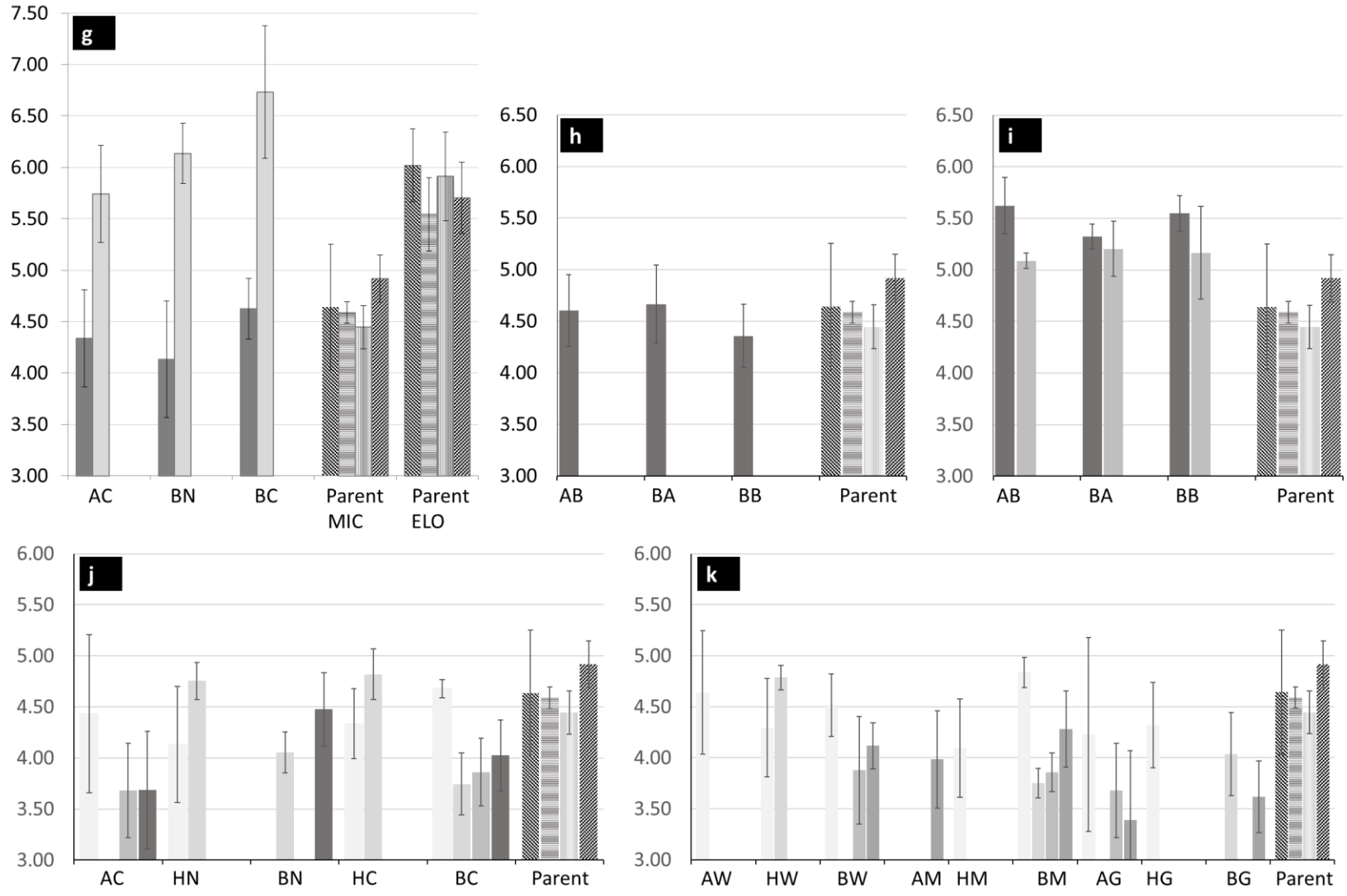
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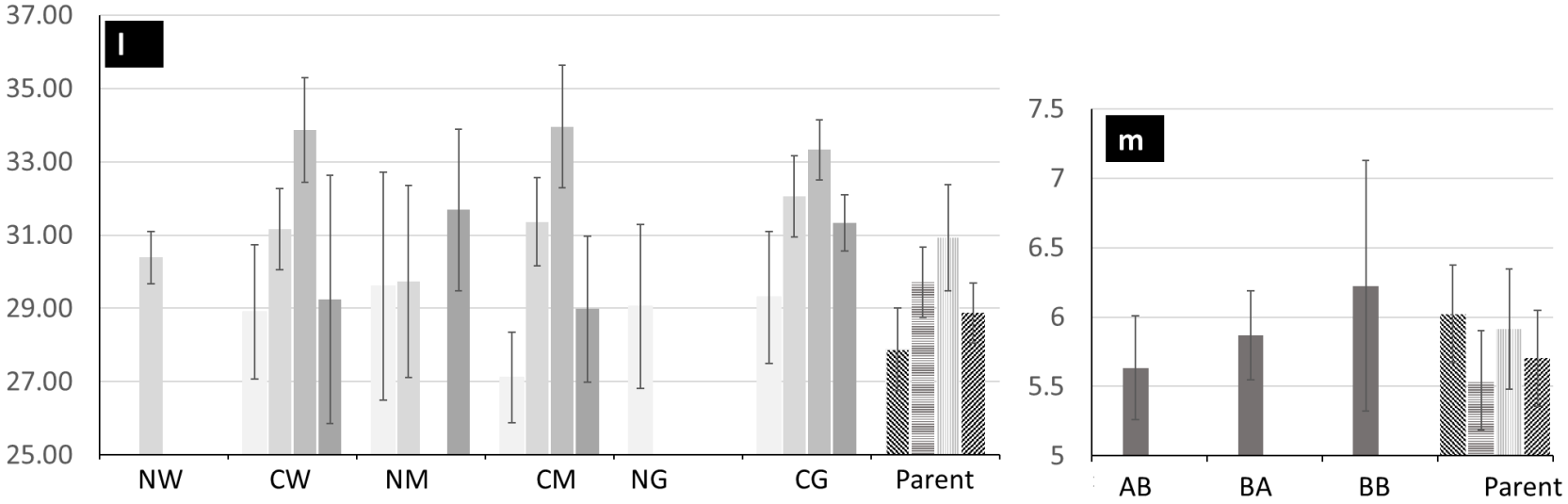
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CHAPTER 6

DISSECTING BIOMETRIC PARAMETERS OF FIBER QUALITY VARIATION USING GOSSYPIUM TOMENTOSUM INTROGRESSIONS STACKED IN UPLAND COTTON (G. HIRSUTUM L.) [5]

[5] Khanal, S., S, Patel, J.D., Adhikari, J., Chandnani, R., Wang Z., Das, S., Brown N., Jones, D., Chee, P.W., and Paterson, A.H. 2018. To be submitted to *Euphytica*

Abstract

Mendelizing quantitative trait loci (QTL) and identifying 'stable' QTL are prerequisites for marker-assisted selection in crop improvement. We developed QTL-stacked populations in six elite *G. hirsutum* backgrounds targeting one fiber fineness and three fiber elongation QTL introgressed into advanced-backcross *G. hirsutum* lines from *G. tomentosum*. A total of 17 QTL-stacked F_1 individuals were selfed to generate F_2 populations segregating at two to three QTL regions with 33 to 114 plants per population (totaling 1,320 plants), which were field tested for fiber quality traits, genotyped at target QTL regions, selectively advanced to $F_{2:3}$ generation and assessed at two different locations (Athens and Tifton GA). One-way analysis of variance in F_2 populations detected effects of an introgressed QTL region (in chromosome 07) in three different backgrounds (four populations), where *G. tomentosum* QTL conditioned an increase in fiber elongation. Another introgressed region (in chromosome 11) increased fiber fineness (i.e., reduced micronaire) in three populations in GA2004230 background. Correlation among fiber quality traits and favorable shifts in phenotypic means of QTL-stacked populations were partially associated with genotypes at marker loci. Although effects of QTL-stacking were detected in some F_2 populations and selected $F_{2:3}$ lines, QTL main-effects were not uniformly detected across different backgrounds. A digenic combination of *G. tomentosum*-alleles at chromosome 07 and chromosome 14 were putatively associated with fiber elongation in five different *G. hirsutum* backgrounds. Promising QTL targets and improved QTL-stacked lines identified in the current study provide valuable genomic and germplasm resources for molecular breeding to enhance fiber quality traits in Upland cotton.

Introduction

Cotton is the most important natural fiber for the modern textile industry. Commercial production of Upland cotton [*Gossypium hirsutum* L. (AD1)] and Pima cotton [*G. barbadense* L. (AD2)] provide most of the world's textile fibers. Market demands for high quality cotton fiber have generated renewed interest in breeding for better fiber quality (May 1999; Chee and Campbell 2009). However, evolutionary and

breeding bottlenecks have depleted favorable variation within the cultivated gene pool (Paterson et al. 2004; Chee and Campbell 2009) and negative correlation between yield components and fiber quality traits have restricted simultaneous breeding gains. The genetic dissection of complex traits into underlying quantitative trait loci (QTL) creates opportunities to manipulate quantitative traits based on Mendelian principles, using marker-assisted selection (MAS), QTL pyramiding, and map-based cloning, and has potential to mitigate some of the challenges hindering improvement of cotton fiber quality (Chee and Campbell 2009; Fang 2015).

Geneticists have tagged hundreds of QTL underlying cotton fiber quality characteristics (Said et al. 2013; Said et al. 2015a; Said et al. 2015b). Although stability (i.e., consistency) of QTL expression among generations, across environments, and/or among genetic backgrounds has been a major concern (Fang 2015), some QTL show stable and significant genetic effects, suggesting that QTL-MAS can be used to enhance fiber quality traits in Upland cotton (Paterson et al. 2003; Sun et al. 2012; Wang et al. 2012b; Islam et al. 2014;). Successful application of the QTL-MAS in cotton breeding is expected to facilitate simultaneous improvement in lint yield and fiber quality, and to maximize selection gains by facilitating parental selection, reducing phenotyping cost, and expediting generation advance. Both private and public cotton breeding programs are increasingly using molecular breeding strategies to improve cultivated cotton.

Breeders have routinely introduced and shuffled exotic alleles from primitive *G. hirsutum* and *G. barbadense* accessions in attempts to improve modern varieties (McCarty et al. 1995; McCarty et al. 2004; McCarty et al. 2007; Zhang et al. 2014), with increasing opportunity for application of molecular genomic tools based on published marker-trait associations (Cao et al. 2014; Said et al. 2015a; Adhikari et al. 2017; Chandnani et al. 2017b; Chandnani et al. 2017c). Systematic introgressions from *G. tomentosum* Nutall ex Seemann (AD3), *G. mustelinum* Miers ex Watt (AD4), and *G. darwinii* Watt (AD5) have recently been explored (Waghmare et al. 2005; Zhang et al. 2011; Wang et al. 2012a; Chen et al. 2015; Oluoch et al. 2016) to introduce rich novel allelic variation into the cultivated gene pool. Interspecific crosses between

G. hirsutum and *G. tomentosum* have been used for F_2 mapping (HT or HM map) (Waghmare et al. 2005), backcross-selfed mating design QTL analysis (Zhang et al. 2011), and chromatin transmission study (Waghmare et al. 2016). Despite the extremely poor fiber quality of the wild species, transgressive segregation in fiber quality was traced to favorable alleles from the wild, attesting to assertions that seemingly unfavorable parents could contribute 'stable' QTL with significant phenotypic effects (Paterson et al. 1988; Tanksley et al. 1996). A number of favorable QTL alleles were contributed by *G. tomentosum* for fiber elongation and fineness - each trait with relatively short history of selection in scientific cotton breeding (Zhang et al. 2011).

Backcross-selfed introgression lines developed from HT study carry favorable interspecific alleles in a near-isogenic state, for which 'stability' needs to be assessed in different genetic backgrounds, over different generations, and preferably under different environments. The objective of this research is to determine biometric parameters of QTL-MAS employed to assess four *G. tomentosum* QTL transferred from near-isogenic introgression lines to secondary segregating populations in genetic backgrounds of elite *G. hirsutum* lines representative of US 'cotton belt'. Specifically, three fiber elongation (qFE11.1, qFE14.1, and qFE21.1), and one fiber fineness (qFF07.1) QTL alleles were targeted for developing 17 QTL-stacked secondary segregating populations (F_2 and $F_{2:3}$) to validate individual and interactive QTL effects and 'stability' in genetic backgrounds of *G. hirsutum*.

Materials and Methods

Plant Materials and Population Development

Genetic materials and the population development approach used in generating secondary segregating populations used in the current study is illustrated in **Figure 6.1**. The target QTL, chromosomal affiliations, diagnostic RFLP loci, and pertinent QTL information are presented in **Supplemental File S6.01**. In summary, *G. hirsutum* x *G. tomentosum* (HT) advanced-backcross lines (BC_3F_2) carrying a fiber fineness (qFF-7-1) and three fiber elongation QTL (qFE11-1, qFE-14-1, and qFE-21-1) (herein referred to

as *Gt*-QTL) were identified from Zhang et al. (2011), retrieved from UGA-PGML (http://www.plantgenome.uga.edu/), and advanced to $BC_3F_{2:3}$ generation in 2011. Selected $BC_3F_{2:3}$ lines with *Gt*-QTL were crossed with six *G. hirsutum* elite lines representing four cotton growing regions of the United States (i.e., GA2004230, GA2004089, and R01-40-08 representing the Southeastern US, Deltapine DP 50 representing the Mississippi Delta, Paymaster HS 26 representing the Southern Plains, and Acala SJ4 representing the Southwestern region(s) of the US cotton belt).

Gt- QTL selections were planted in crossing blocks at the UGA Plant Science Farm, Watkinsville, GA in 2012, where *Gt*-QTL x *Gt*-QTL crosses within the elite *G. hirsutum* backgrounds were attempted in all possible combinations. First generation hybrids from successful crosses were grown in a greenhouse using seedling containers filled with commercial garden soil mix. Diagnostic SSR markers were used to genotype *Gt*-QTL x *Gt*-QTL crosses (20 individuals each when available) at target regions to identify QTL-stacked lines, which were transferred to bigger pots and selfed to develop F_2 populations. A few F_2 lines from each population were selectively advanced to $F_{2:3}$ generation based on genotypes at diagnostic marker loci. Specifically, individuals homozygous at one or more putative QTL were advanced to multilocational replicated trials (discussed later).

Marker Resources

Gt-QTL targets were originally mapped and delineated with diagnostic RFLP markers (Zhang et al. 2011) (**Supplemental File S6.01**). Since the HT map was constructed from a subset of RFLPs in a composite HB map (Rong et al. 2004), heterologous bridge loci including orthologs of diagnostic RFLPs could be traced in the HB map, which then was juxtaposed with SSR-rich maps [HM map (Wang et al. 2016b), HB maps (Guo et al. 2007; Yu et al. 2007), and a comprehensive reference map (Yu et al. 2010)]. Markers potentially spanning *Gt*-QTL targets were identified, and oligonucleotide primers [synthesized by Eurofins MWG Operon (Huntsville, AL)] were used to screen *G. hirsutum* (var. CA3093) and *G. tomentosum* (acc. AD3-16) for polymorphisms. Polymorphic markers were further screened against a small

subset of the HT population to ascertain linkage with diagnostic RFLP loci and putatively linked SSRs were used for marker-assisted selection (MAS) during population development. R01-40-08 is a NIIL homozygous for *G. barbadense* cv. Pima S6 chromatin segment carrying a fiber length QTL qFL-chr1, which conditions longer fiber in the recurrent background of *G. hirsutum* cv. Tamcot 2111 (Shen et al. 2011). Markers reportedly spanning qFL-chr1 were used to track *G. barbadense* alleles.

Segregation ratios at diagnostic marker loci [i.e., 1:2:1 (two homozygotes and a heterozygote) for a codominant marker and 3:1 (presence vs absence) for a dominant marker] and deviations from independent assortment of alleles at two unlinked loci were tested for the goodness of fit using Pearson's chi squared tests ($P = 0.01$).

Wet Lab Protocols

Genomic DNA was extracted using a modified cetylmethylammonium bromide (CTAB) method (Paterson et al. 1993). A single, unfolded, and preferably second true leaf was collected from individual plants early in the morning. An Omni Bead Ruptor 24 Homogenizer (Omni International) was used for grinding the leaf tissues, and nuclei extraction and nuclei lysis buffer were added as a 1:1 solution. Downstream procedures followed the established protocol with an adjustment in volumes of added solutions for the mini-prep. The quality and quantity of genomic DNA was assessed with a NanoDrop 2000 (Thermo Scientific). PCR reactions and procedures to resolve amplified fragments followed our standard protocols (Khanal et al. 2017). Some of genotyping involved fluorescently labelled primers, modified PCR protocol, and analysis using an ABI 3730 automatic DNA Analyser (available upon request).

Field Trials and Phenotyping

QTL-stacked F_2 populations and 5 parental replicates (in 1m plots) were hand planted at the Plant Science Farm, Watkinsville, GA in 2013. Agronomic practices including plant spacing, irrigation, fertilization, and pest control followed commercial recommendations. Fiber samples were hand-picked from 15 open bolls per plant (when available). Samples were ginned using an 8-saw gin and 10 grams of

lint were sent to the Cotton Incorporated Textile Services Laboratory (Cotton Inc., Cary, NC) where six standard fiber quality parameters including fiber fineness or micronaire (MIC), upper half mean fiber length (UHM), fiber length uniformity (UI), fiber strength (STR), fiber elongation (ELO), and short fiber content (SFC%) were determined using a High-Volume Precision Instrument (HVI; Zellweger-Uster, Knoxville, Tenn.). Populations with poor stands (due to poor germination) were replanted at the same location in 2014, and fiber properties were determined as discussed above.

Selected $F_{2:3}$ lines and parental replicates were hand planted in 1m plots (spaced 1m apart) in randomized complete blocks (2 replicates) at two locations (Plant Science Farm at Watkinsville, GA; and Gibbs farm, Tifton, GA) in 2014. A total of 25 boll samples were hand-harvested from each plot, ginned using a 12-saw gin, and fiber phenotype (HVI) for standard fiber quality parameters were obtained from HVI analysis at Cotton Inc.

Phenotypic Analysis of F_2 and $F_{2:3}$ Trials

JMP Pro was used to compute population distributions statistics (mean, standard deviations) and Pearson's correlation coefficients, and to perform ANOVA (one-way and two-way) and mean comparison procedures. Specifically, LSD test employing Student's t-tests, Tukey-Kramer HSD, and With Control, Dunnett's tests (against parental controls) were executed with significance declared at alpha level of 0.05. Partitioning of phenotypic variances into genotype (G), environment (E), and genotype x environment (G x E) interactions among parental lines, selected F_2 individuals, and their $F_{2:3}$ progenies were carried out using standard least squares models that perform two-way ANOVAs, with significance reported at $p < 0.05$.

Marker-trait Associations at Diagnostic SSR loci

JMP Pro was used to identify marker-trait associations (Student's *t*-test, ANOVA), using a relaxed single-comparison confidence level (CL) of 95% ($p < 0.05$), with consideration that our inferences were primarily based on genotypes at few predetermined marker-tagged QTL-introgressed sites and that observed associations were further assessed among different populations across four to six genetic

backgrounds, and in $F_{2:3}$ multilocational trials. Hence, marginal associations (i.e., $0.01 > p < 0.05$) were discussed to evaluate stability and replicability of potential QTL. The percentage of total phenotypic variance explained (R^2), QTL effects (additive effect of an allele substitution), and significance of digenic interactions (i.e., haplotype at two loci influencing response trait phenotypes) were obtained from JMP Pro. Gene action (additivity and dominance) was computed as described (Paterson et al. 1990).

For loci segregating in two or more populations of one background, or in multiple backgrounds, two-way ANOVA was performed using the standard least squares 'Fit Model' function employed in JMP Pro. The analytical model included genotype (G), family (F) and genotype x family (G x F) interaction or background (B) and genotype x background (G x B) interaction as fixed factors. Genotypic effects and interaction effects were considered significant at $p < 0.05$. Similarly, effect of QTL-stacking was assessed based on the 'Fit Model' function to test for the significance of main-effects and interactions at diagnostic SSR marker loci, with significance declared at $p < 0.05$. Effects of QTL-stacking were further assessed based on ad hoc means comparisons among $F_2/F_{2:3}$ lines and *G. hirsutum* parents in their immediate pedigrees, using pooled phenotypes over generations and locations for different genotypic classes and parental samples.

Results

MAS towards QTL-stacked Populations

SSR markers were used to identify advanced-backcross lines carrying introgressed QTL and for MAS to develop QTL-stacked populations. Four *Gt*-QTL were tagged with one to three SSR markers each, whose veracity was supported by genetic linkage to diagnostic RFLPs and/or reported allele size information. A total of 17 QTL-stacks in six *G. hirsutum* elite lines and cultivars were developed, and individuals heterozygous at target QTL were selfed to constitute F_2 populations for QTL mapping in early segregating generations. Twelve populations were grown in 2013 while five were grown in 2014. F_2 lines

from 2013 F_2 populations were selectively advanced to $F_{2:3}$ generation and evaluated in 2014. Details of QTL mapping populations are presented in **Table 6.1**.

Alleles at the diagnostic loci mostly exhibited normal segregation in F_2 populations, with only two cases showing significant ($P < 0.01$) distortions [i.e., NAU1014 (chromosome 11) in pop. 05 and NAU2998 (chromosome 21) in pop. 02]. The distortion in pop. 05 led to significant deviations from expected ratio (i.e., 9:3:3:1) of independent assortment of alleles of NAU1014 (chromosome 11) and NAU3731 or NAU3074 (chromosome 21).

Phenotypic Distribution and Means Comparison

The distribution of fiber quality characteristics in F_2 individuals is shown in **Table 6.1** and histograms corresponding to MIC and ELO distributions are presented in **Supplemental File S6.02**. F_2 populations displayed wider distributions of MIC and ELO phenotypes than parental genotypes, suggesting positive and negative transgression.

G x E interactions were not significant (at $p < 0.0001$), allowing us to pool data from selected F_2 and $F_{2:3}$ lines across environments (data not shown). In general, background-specific means of F_2 populations and selected $F_2/F_{2:3}$ lines compared to parental controls showed a shift towards favorable values for MIC and ELO - the two traits for which QTL were introduced. On the contrary, there was a lack of favorable shift and even significant decline in the other four fiber quality traits measured.

Micronaire (MIC) Five populations, including one each from GA2004089 (pop. 04) and Paymaster HS 26 (pop. 02), and three from R01-40-08 (pop. 01, pop. 06, and pop. 07), showed significantly lower (improved) average MIC values compared to appropriate parental checks, whereas one population in GA2004230 background (pop. 16) had significantly higher MIC. In pooled analysis, selected $F_2/F_{2:3}$ progenies from pop. 04 and one pop. 09 selection (249-089) showed significantly lower MIC values, while all other selections were not significantly different from their appropriate parental checks.

Fiber Elongation (ELO) Six populations had higher average ELO (improved), which included one Acala SJ4- (pop. 10), two GA2004230- (pop. 03 and pop. 16), and three R01-40-08-populations (pop. 01, pop. 08, and pop. 09). In pooled analysis, selections from pop. 10 in Acala SJ4, pop. 14 in Deltapine DP 50, pop. 03 and pop. 11 in GA2004230, and pop. 07, pop. 08 and pop. 09 in R01-40-08 backgrounds showed significantly higher ELO values compared to the parental lines.

Fiber strength (STR), fiber length (UHM), length uniformity (UI), and short fiber content (SFC%) In general, average STR, UHM, and UI of F_2 populations and $F_{2:3}$ selections were either comparable or inferior to respective parental controls. Similarly, average SFC% were mostly comparable and, in a few cases, significantly higher (inferior) in both generations.

Correlation between Traits

Significant correlations among fiber quality traits corroborate frequently reported associations; specifically, MIC -- ELO and UHM -- STR were positively correlated, while traits across groups (e.g., MIC or ELO against UHM or STR) showed negative associations - the trend was apparent in F_2 populations and in pooled $F_2/F_{2:3}$ selections (**Supplemental File S6.05**). Correspondence between selected F_2 and $F_{2:3}$ for the six fiber quality traits were positive and significant ($r = 0.42$ to 0.78, $p < 0.001$).

Marker-trait Associations at Diagnostic SSR Loci

Biometric summary of marker-trait associations, *G* x *B* (or *G* x *P*) interactions, and significant digenic interactions influencing six fiber quality characteristics are presented in **Table 6.2**, **Supplemental File S6.06**, and **Supplemental File S6.07**, respectively. Similarly, biometric summary of pooled analysis among $F_2/F_{2:3}$ selections are presented in **Supplemental File S6.08** and **Supplemental File S6.09**. Partial summary of QTL-specific observations from the current study follows:

Gt-QTL qFF07.1

NAU2432 was linked to diagnostic RFLP locus G115bE3C for fiber fineness QTL (qFF07.1) introgressed from *G. tomentosum* into chromosome 07 of *G. hirsutum* cultivar CA3093. BC_3F_3 individuals from lines showing *Gt*-introgression at the locus were used in MAS for developing nine populations, which represent six different *G. hirsutum* backgrounds. Of populations segregating at the diagnostic SSR locus, *Gt*-alleles were associated with decreased MIC (R^2 = 0.21 to 0.30) in two GA2004230-populations (pop. 11 and pop. 16), with ANOVA showing significant population effect ($p < 0.0001$) and *G* x *P* interaction ($p < 0.016$). However, decreased MIC associated with *Gt*-alleles at NAU2432 was not detected in pooled analysis of $F_2/F_{2:3}$ selections.

In a companion study, *Gt*-alleles of NAU2432 were putatively associated with increased ELO in four different populations in two *G. hirsutum* backgrounds (*Gt*-QTL x *Gm*-QTL; our unpublished study). In the current study, *Gt*-alleles of NAU2432 increased ELO in four different F_2 populations (pop. 08, pop. 11, pop. 12, and pop. 13), in three different backgrounds (GA2004230, R01-40-08, and Paymaster HS 26) including additional populations corresponding to the two backgrounds used in the companion study (GA2004230 and R01-40-08), suggesting that the region harbors a major gene(s) associated with fiber elongation. ANOVA showed significant background and population effects on ELO, but their interactions with genotype were not significant. Contrasts between genotypes were not significant in $F_2/F_{2:3}$ selections.

Gt-QTL qFE11.1

A fiber elongation QTL on chromosome 11 (qFE11.1) was tagged with three SSR markers viz. NAU1014, NAU5212, and BNL2632, based on their linkages with the diagnostic RFLP locus pBAM422yE3C. BC_3F_3 individuals showing *Gt*-introgression at the locus were used in MAS for developing nine F_2 populations. In our companion study (*Gt*-QTL x *Gm*-QTL; our unpublished data), NAU1014 was associated with a genetically dominant increase in ELO in only one of the six QTL-stacked populations. Further, *Gt*-alleles from NAU1014 also showed significant among family genotypic effect between two F_2

populations, resulting from decreased MIC values. In the current study, an additional nine populations were studied, where dominance effect at the diagnostic SSR locus increased fiber elongation in one population (pop. 02). Consistent with prior findings, ANOVA showed significant background effect and among background G effect at NAU1014, where *Gt*-alleles decreased (improved) MIC in three different GA2004230-populations (pop. 03, pop. 05, and pop. 11). Contrasts between $F_2/F_{2:3}$ genotypic classes were not significant for both MIC and ELO.

Gt-QTL qFE14.1, qFF14.1, and qFU14.1

We identified amplicons corresponding to a codominant marker, NAU5027, that was linked to RFLP locus pAR815E3C associated with qFE14.1, qFF14.1, and qFU14.1. Increased ELO was conditioned by *Gt*-alleles, while the locus showed significant genotype x family (G x F) interaction for fiber fineness and uniformity. Following QTL-MAS, backcross-selfed lines were used to develop nine segregating populations representing five *G. hirsutum* backgrounds. ANOVA showed significant differences in ELO among backgrounds/populations, while among background/population G effect was also significant. Contrasts among genotypes in individual populations were not significant, suggesting that the G effect was only significant in models where background/population were included. Pooled analysis also showed significant among backgrounds (and populations) as well as significant G effect at the locus, with two populations (pop. 08 and pop. 10) reaching statistical significance for contrasts between genotypes carrying homozygous *Gt*-alleles versus *Gh*-homozygotes, the former increasing fiber elongation by 8% to 17%.

Gt-QTL qFE21.1 and qFF21.1

Three markers viz. NAU2998, NAU3074, and NAU3731 were linked to the RFLP locus G1261aE3C associated with a fiber elongation (qFE21.1) and a fiber fineness QTL (qFF21.1), at which the *Gt*-allele mapped to chromosome 21 contributed favorable phenotypic effects. Of seven new populations in four *G. hirsutum* backgrounds, *Gt*-alleles at the diagnostic SSR markers were associated with increased fiber elongation in one population (pop. 13); increased UHM in two populations (pop. 04 and pop. 05) and

increased UI in two populations (pop. 02 and pop. 05). However, contrasts among $F_2/F_{2:3}$ genotypes were not significant for these increases.

Gb-QTL qFL-chr1

A total of four populations carried the *Gb*-allele of marker JESPR56, associated with fiber length QTL qFL-chr1 (Shen et al. 2011). Marker-trait association was detected in one F_2 population (pop. 09), while the average length of pop. 07 was not significantly different from R01 parental check. Contrasts between selectively advanced $F_{2:3}$ genotypes were not significantly different, but most selections from pop. 01, pop. 07, and pop. 08 had comparable fiber lengths with R01-40-08, while those of pop. 09 had significantly lower lengths.

Consistency of QTL Across Populations

A total of 72 two-way ANOVAs were performed for diagnostic SSR loci segregating in two or more F_2 populations (within backgrounds) to assess the consistency of marker-trait associations. A significant ($p < 0.05$) among-family genotype effect was detected in 14 (19.4%) cases, eight in GA230- and six in R01-populations (**Supplemental Table S6.06**), while significant G x P (among backgrounds) interactions were present in three (4.2%). These loci represent four nonoverlapping genomic regions, one each in chromosomes 01, 07, 11, and 21. Pooled analysis of $F_2/F_{2:3}$ lines showed significant among-population genotypic effects (G) of NAU2432c for UHM (**Supplemental Table S6.08**), an observation consistent with F_2 analysis.

Effects of QTL Stacking

A total of 312 two-way ANOVAs were performed to detect main-effects of stacked QTL loci and their digenic interactions. A total of four (1.2%) models were significant ($p < 0.05$) including one constituting significant digenic interaction (**Table 6.2; Supplemental Table S6.07**). Only one of the three models without interaction [constituting *Gt*-alleles at NAU2432 (chromosome 07) and NAU3074

(chromosome 21)] showed favorable effect of allele stacking, increasing ELO in pop. 13, while another model [constituting *Gt*-alleles at NAU2432 (chromosome 7) and NAU5027 (chromosome 14)] showed unfavorable effect, decreasing fiber length uniformity (UI) in pop. 12.

Further, ad hoc means comparison (see 'Materials and Methods') was employed to quantify effects of QTL-stacking using 240 digenic combinations of stacked alleles in $F_2/F_{2:3}$ selections from 12 populations. Although phenotypic effects of allele stacking were not uniform, contrasts among different combinations of stacked alleles in selected lines from pop. 08 (R01-40-08 background), pop. 10 (Acala SJ4 background) and pop. 12 (Paymaster HS 26 background), segregating at two codominant marker loci [NAU2432 (chromosome 07) and NAU5027 (chromosome 14)], indicated that the two unlinked loci show digenic interaction. For example, significant main-effects at NAU2432 were associated with increased ELO in F_2 populations (pop. 08 and pop. 12) (**Table 6.2**), while contrasts between homozygous selections (i.e., *Gh*-alleles vs *Gt*-alleles) were significant in pooled $F_2/F_{2:3}$ (pop. 08 and pop. 10) with increased elongation conditioned by *Gt*-alleles at NAU5027 (**Supplemental Table S6.08**). In pooled analysis of pop. 08 and pop. 10 selections, doubly homozygous lines showed 2-15% and 18-27% higher ELO over singly homozygous lines and 29% and 49% increase compared to R01-40-08 and Acala SJ4 parents, respectively (**Figure 6.2a**). On the contrary, doubly homozygous selections of pop. 12 showed 6-8% decrease in fiber elongation compared to the singly homozygous lines and 3% decrease in ELO compared to Paymaster HS 26 parent (**Figure 6.2a**). While the phenotypic effects of QTL stacking were not uniform across backgrounds, NAU2432-NAU5027 stacking putatively increased ELO in four of five different populations/backgrounds [i.e., pop. 08 (R01-40-08), pop. 10 (Acala SJ4), pop. 16 (GA2004230), pop. 17 (GA2004089)], with concurrent decline in other fiber characteristics (except SFC%) (**Figure 6.2, Supplemental File S6.10**) and warrant further investigation.

Discussion

Although Upland cotton cultivars have a narrow genetic base, breeders have routinely introduced novel allelic variation via introgression breeding into Upland germplasm, a portion of which is quantitative in nature and has also been Mendelized through AB-QTL (Tanksley et al. 1996) and backcross-selfed (Chee et al. 2005b) approaches. QTL showing favorable phenotypic effects and stable expression are promising targets for molecular breeding. Several research and breeding efforts are underway that build upon early QTL studies, particularly to validate detected QTL in advanced generations, different genetic backgrounds, and/or different environments. In the current study, we developed and tested 17 early generation segregating populations (i.e., F_2 and $F_{2:3}$) to further assess QTL effects and 'stability' of four fiber quality QTL introduced into *G. hirsutum* from *G. tomentosum* (Waghmare et al. 2005; Zhang et al. 2011; Waghmare et al. 2016). A MAS-QTL approach was used to stack fiber elongation and fiber fineness QTL in different combinations (**Supplemental File S6.01**) in four to six different *G. hirsutum* genetic backgrounds representing four growing regions of the United States cotton belt. Several F_2 populations and selected $F_{2:3}$ lines showed favorable shifts in average phenotypes for MIC and ELO, the two traits specifically targeted in the current study.

Average phenotypes of six F_2 populations [pop. 10 (Acala SJ4), pop. 03 and pop. 16 (GA2004230), and pop. 01, pop. 08 and pop. 09 (R01-40-08)] in three different backgrounds showed significant increases in ELO compared to corresponding parental backgrounds (**Table 6.1**; **Supplemental File S6.03**). Selectively advanced lines from four (pop. 03, pop. 08, pop. 09 and pop. 10) of the six populations and selections from three additional populations [pop. 07 (R01-40-08), pop. 11 (GA2004230) and pop. 14 (Delta Pine DP 50)] had higher average ELO than respective parents in pooled analysis of $F_2/F_{2:3}$ data (**Supplemental File S6.04**). Interestingly, all populations and/or selections that showed significant improvement over appropriate parental background carried *Gt*-introgressions at chromosome 07 and/or chromosome 14, supporting the veracity of significant marker-trait associations detected at NAU2432

(chromosome 07; **Table 6.2**) in four F_2 populations [pop. 08, pop. 11, pop. 12 (Paymaster HS 26) and pop. 13] and significant among population genotypic effect at NAU5027 (chromosome 14) in pooled analysis of $F_2/F_{2:3}$ selections (**Supplemental File S6.09**). In our companion study (*Gt*-QTL x *Gm*-QTL; our unpublished data), *Gt*-alleles at NAU2432 were putatively associated with increased ELO in four F_2 populations and/or selected $F_2/F_{2:3}$ lines in GA2004230 and R01-40-08 backgrounds. Further, we found that *Gt*-alleles at NAU5027 were nominally associated ($p < 0.05$) with ELO in two different populations. However, ELO QTL associated with NAU2432 and NAU5027 were mostly detected only at liberal significance thresholds ($p < 0.05$) in both of our companion studies. Small effect size at these QTL appears plausible given that most *Gt*-QTL contributed relatively small proportions of overall phenotypic variation in fiber quality characteristics (Zhang et al. 2011). Further, *Gt*-QTL targeted in the current study explained 4.5-9.0% variation in fiber phenotype in a growout at Lubbock, Texas (Zhang et al. 2011). Specifically, significant variation was explained by qFE14.1 and qFE21.1 in BC_3F_2 (R^2=7.8-9%) and BC_3F_3 (R^2=4.5-5.7%); and by qFE11.1 and qFF07.1 in BC_3F_2 (R^2=6.7%) and BC_3F_3 (R^2=6%) generations, respectively, with each QTL reaching significance only at Lubbock. Nevertheless, results of the current study and our companion study (unpublished) indicate that favorable small effects at NAU2432 and NAU5027 were transferable to different genetic backgrounds in a different environment. The current study, using early generation segregants and smaller populations to decipher downstream utility values is encouraging but merits further assessment.

Genetic studies have underscored the importance of epistasis as a basis of heterotic responses in cotton. For example, significant epistatic interactions have been reported for cotton morphological and phenological traits (Guo et al. 2008; Song and Zhang 2009; Liang et al. 2014; Liu et al. 2014), yield and yield components (Jia et al. 2014), insect-pest and disease resistance (Ulloa et al. 2010; He et al. 2014), and fiber quality traits (He et al. 2008; Saha et al. 2011; Cao et al. 2014; Guo et al. 2014; Yuan et al. 2014; Song et al. 2017). One significant digenic interaction was detected in the current study, and five in our companion study (unpublished). Since individual fiber quality QTL often contribute relatively little to overall

phenotypic variation (<10%) of a trait (Said et al. 2015b; Jamshed et al. 2016) and may be exacerbated by profound environmental influence (Paterson et al. 2003; Lacape et al. 2010), QTL pyramiding is attractive for achieving commercially important levels of improvement in fiber quality traits of Upland cotton.

QTL-MAS pyramids targeting fiber quality traits in Upland cotton have mostly focused on fiber length (Souder et al. 2004; Dong et al. 2009) and fiber strength (Yi et al. 2004; Guo et al. 2005; Yuan et al. 2014). For example, Yuan et al. (2014) pyramided two fiber strength QTL and showed that stacked lines had higher fiber strengths than lines carrying single QTL. Similarly, Cao et al. (2014) developed improved QTL-stacked lines carrying *G. barbadense* introgression associated with increased fiber length, strength, and micronaire. Guo et al. (2014) also detected and performed MAS of three QTL (one each for fiber length, strength, and micronaire) where favorable alleles were contributed by *G. barbadense*. In our companion study, we detected enhanced fiber properties associated with some QTL-stacks, where doubly homozygous $F_2/F_{2:3}$ lines showed significantly higher elongation than singly homozygous lines and parental checks. In the current study, we found a few QTL-stacks with increased trait value. For example, selectively advanced QTL-stacked (doubly homozygous) $F_2/F_{2:3}$ lines of pop. 10 showed significantly higher elongation than singly homozygous lines and parental checks, which supports the merit of QTL-MAS pyramiding for increased fiber elongation in Upland cotton.

Correlations observed among fiber quality traits were generally in line with prior reports (Lacape et al. 2005; Shen et al. 2007) and co-localization of QTL underlying different fiber quality traits partially explained moderate to strong correlation among fiber phenotypes. For example, significant positive correlations between UHM and STR in pop. 04 and pop. 05 were partially explained by *Gt*-introgressions at chromosome 21 (NAU3074 and NAU3731). Pleiotropic or close linkage relationships among fiber quality QTL were also reported in *G. tomentosum* (HT) studies. For example, an ELO QTL (qFE14.1) co-located with a fiber fineness (qFF14.1) and an UI (qFU14.1) QTL in HT populations, with *G. tomentosum* alleles conferring favorable ELO and MIC (fineness). qFF14.1 and qFU14.1 showed significant G x B

interactions, and both were detected in *G. hirsutum* x *G. barbadense* F_2 populations (Paterson et al. 2003). We also detected a significant main effect of qFU14.1 in pop. 12, while contrast between $F_2/F_{2:3}$ selections (pop. 08 and pop.10) homozygous for *Gh*- and *Gt*-alleles at the locus were significant for ELO. Interestingly, *Gt*-introgressions at two QTL targets, viz. qFF07.1 for MIC and qFE11.1 for ELO, were found to be associated with ELO and MIC, respectively. These cases support reported findings that co-localized/common genetic loci may affect correlated traits (Chee et al. 2005b).

Fiber quality QTL can express erratically across different environments, generations, and/or genetic backgrounds (Paterson et al. 2003; Sun et al. 2012; Fang 2015; Jamshed et al. 2016; Liu et al. 2017). Our assessment of four exotic QTL alleles using QTL-stacked populations (i.e., *Gt*-QTL x *Gt*-QTL) in Upland cotton backgrounds representing four major cotton production regions of the US cotton belt finds *G. tomentosum* introgressions at chromosome 07, chromosome 11 and chromosome 14 promising for QTL-pyramiding to enhance fiber quality. QTL-stacked germplasm developed in this study will allow *G. tomentosum* alleles to be more readily accessible to regional breeding programs and provides a foundation for using QTL-stacked lines to develop superior QTL-pyramids.

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Table 6.1 Distribution of average fiber quality phenotypes of parental checks and QTL-stacked populations in genetic backgrounds of *G. hirsutum*.

Plant materials	Epithet	Year	Background	Specifics[a]	Size[b]	Fiber quality phenotypes[c]					
						Micronaire (MIC)	Upper Half Mean Length UHM (inch)	Length Uniformity Index UI (%)	Fiber Strength STR (gm/tex)	Fiber Elongation ELO (%)	Short Fiber Content SFC (%)
Parental checks											
CA3093	CA93 (2013)	2013		Check; recurrent parent of *Gt*	8	4.86±0.34	1.02±0.03	83.19±1.18	27.05±1.24	6.14±0.31	7.45±0.61
CA3093	CA93 (2014)	2014		QTL introgressions	5	5.11±0.43	1.04±0.06	82.68±1.06	27.84±1.01	6.18±0.39	7.86±0.63
Acala SJ4	AC4 (2013)	2013		Parental check	8	4.70±0.42	1.12±0.03	85.20±0.91	29.49±1.94	4.79±0.50	6.68±0.17
Acala SJ4	AC4 (2014)	2014			4	4.39±0.17	1.12±0.03	82.98±0.57	29.53±0.83	4.78±0.47	8.03±0.49
Deltapine DP 50	DP50 (2013)	2013		Parental check	8	4.74±0.16	1.11±0.03	83.28±1.37	26.11±0.67	6.44±0.61	7.85±0.45
Deltapine DP 50	DP50 (2014)	2014			5	4.68±0.89	1.07±0.03	82.14±1.14	27.02±1.55	6.08±0.50	8.90±1.21
GA2004230	GA30 (2013)	2013		Parental check	18	4.57±0.25	1.15±0.04	84.33±1.05	29.01±1.30	5.54±0.33	7.21±0.43
GA2004230	GA30 (2014)	2014			9	4.71±0.23	1.16±0.05	83.76±0.97	29.30±1.04	5.78±0.53	7.69±0.23
GA89	GA89 (2013)	2013		Parental check	8	4.36±0.32	1.12±0.06	84.59±1.11	28.66±1.64	6.11±0.43	7.25±0.56
GA89	GA89 (2014)	2014			5	4.70±0.42	1.15±0.04	84.32±0.88	30.96±1.54	6.24±0.38	7.38±0.26
Paymaster HS26	PM26 (2013)	2013		Parental check	8	4.91±0.15	1.06±0.02	83.25±1.51	28.41±1.12	6.69±1.23	6.85±0.60
Paymaster HS26	PM26 (2014)	2014			5	4.91±0.18	1.04±0.02	82.96±0.94	28.42±1.20	5.80±0.34	8.30±0.47
R01-40-08	R01 (2013)	2013		Parental check	16	4.34±0.57	1.18±0.04	84.89±0.87	30.42±2.04	6.25±0.52	7.03±0.42
R01-40-08	R01 (2014)	2014			10	4.68±0.37	1.14±0.04	83.55±1.45	29.92±1.44	5.99±0.79	7.86±0.79
QTL-stacked populations											
Pop. 01		2013	R01	*Gt(11)xGt(14)xGb(01)*	109	3.66±0.68	1.10±0.06	82.94±1.56	26.48±2.03	6.79±0.80	8.33±1.03
Pop. 02		2013	PM26	*Gt(11)xGt(21)*	98	4.28±0.73	1.00±0.05	82.46±1.27	25.24±1.65	5.52±0.56	8.49±1.00
Pop. 03		2013	GA30	*Gt(11)xGt(14)*	62	4.42±0.46	1.04±0.05	83.57±1.27	26.98±1.41	6.64±0.62	7.39±0.79
Pop. 04		2013	GA89	*Gt(11)xGt(21)*	73	3.75±0.36	1.10±0.05	83.89±1.33	27.58±1.73	5.86±0.65	7.48±0.68
Pop. 05		2013	GA30	*Gt(11)xGt(21)*	77	4.55±0.45	1.10±0.05	84.22±1.08	27.02±1.56	5.48±0.46	7.28±0.60
Pop. 06		2014	R01	*Gt(11)xGt(21)*	39	3.07±0.44	1.16±0.04	81.88±1.32	27.97±1.42	5.88±0.45	9.15±0.91
Pop. 07		2013	R01	*Gt(14)xGt(21)xGb(01)*	107	3.95±0.42	1.18±0.05	84.18±1.38	28.98±1.84	6.63±0.76	7.58±0.68
Pop. 08		2013	R01	*Gt(07)xGt(14)xGb(01)*	97	4.02±0.60	1.12±0.06	83.15±1.18	26.42±1.74	7.62±0.93	7.90±0.63
Pop. 09		2013	R01	*Gt(07)xGt(11)xGb(01)*	114	4.47±0.42	1.05±0.06	83.55±1.25	26.45±1.95	7.41±0.71	7.55±0.78
Pop. 10		2013	AC4	*Gt(07)xGt(14)*	68	4.64±0.56	1.02±0.04	83.81±1.00	27.41±1.57	5.96±0.70	7.08±0.74
Pop. 11		2013	GA30	*Gt(07)xGt(11)*	74	4.53±0.56	1.02±0.05	82.70±1.30	24.82±1.49	5.84±0.73	8.33±1.03
Pop. 12		2013	PM26	*Gt(07)xGt(14)*	114	4.88±0.48	1.06±0.05	83.77±1.11	28.51±2.17	6.05±0.73	7.22±0.71
Pop. 13		2014	GA30	*Gt(07)xGt(21)*	48	4.72±0.40	1.11±0.04	83.57±1.39	26.98±1.77	6.20±0.70	7.96±0.87
Pop. 14		2013	DP50	*Gt(07)xGt(11)*	93	4.40±0.62	1.02±0.05	82.52±1.43	25.71±1.61	6.38±0.72	8.38±1.15
Pop. 15		2014	GA30	*Gt(14)xGt(21)*	54	4.68±0.54	1.10±0.04	83.41±1.16	26.96±1.58	5.91±0.63	8.12±0.78
Pop. 16		2014	GA30	*Gt(07)xGt(14)*	33	5.20±0.33	1.04±0.04	83.08±1.20	27.69±1.40	6.69±0.71	8.16±0.97
Pop. 17		2014	GA89	*Gt(07)xGt(14)*	60	4.95±0.34	1.06±0.03	83.97±0.98	27.47±1.52	6.79±0.78	6.96±0.63

[a] CA3093 is recurrent *G. hirsutum* background of advanced-backcross near isogenic introgression lines (NIILs) carrying *G. tomentosum* (Zhang et al. 2011) QTL. *Gt(07)*, *Gt(11)*, *Gt(14)*, and *Gt(21)* refer to *G. tomentosum* introgressions in chromsomes 7, 11, 14, and 21, respectively. *Gb(01)* refers to *G. barbadense* introgression in chromosome 1. [b] Size references to *G. hirsutum* parental checks denote number of plots, while those for F_2 populations refer to number of individuals in the population. [c] HVI phenotypes.

Table 6.2 Marker-trait associations for fiber quality traits in QTL-stacked *G. hirsutum* F_2 populations.

Trait	Population	Background	Locus	Source	Effect			Test of Significance P > F		R^2
					a	d	d/a	Marker	Model	
Micronaire	Pop. 03	GA2004230	NAU1014c	*Gh/Gt*	-0.29	0.04	-0.16	0.0183		0.11
(MIC)	Pop. 05	GA2004230	NAU1014c	*Gh/Gt*	-0.28	-0.03	0.11	0.0013		0.16
	Pop. 08	R01-40-01	JESPR56c	*Gh/Gb*	0.51	-	-	0.0017		0.11
	Pop. 11	GA2004230	NAU2432c	*Gh/Gt*	0.06	0.39	6.50	0.0060	0.0010	0.30
			NAU1014c	*Gh/Gt*	-0.25	-0.09	0.36	0.0074		
	Pop. 16	GA2004230	NAU2432c	*Gh/Gt*	-0.18	-0.16	0.89	0.0114		0.21
Fiber	Pop. 02	Paymaster HS26	NAU1014c	*Gh/Gt*	-0.11	-0.31	-2.82	0.0164		0.07
Elongation	Pop. 08	R01-40-01	NAU2432c	*Gh/Gt*	-0.17	-0.48	-2.88	0.0260		0.06
(ELO)	Pop. 11	GA2004230	NAU2432c	*Gh/Gt*	-0.35	0.13	0.37	0.0342		0.10
	Pop. 12	Paymaster HS26	NAU2432c	*Gh/Gt*	-0.29	-0.13	-0.44	0.0281		0.05
	Pop. 13	GA2004230	NAU2432c	*Gh/Gt*	-0.33	0.34	1.03	0.0256	0.0116	0.19
			NAU3074c	*Gh/Gt*	-0.06	0.32	-5.03	0.0441		
Fiber	Pop. 09	R01-40-08	NAU1014c	*Gh/Gt*	-0.71	-0.85	-1.20	0.0186		0.06
Strength	Pop. 14	Deltapine DP50	NAU1014c	*Gh/Gt*	-0.77	0.05	0.06	0.0182		0.08
(STR)	Pop. 11	GA2004230	NAU2432c	*Gh/Gt*	-0.76	0.65	0.85	0.0075		0.16
	Pop. 04	GA2004089	NAU3074c	*Gh/Gt*	-0.81	-0.56	-0.70	0.0107		0.10
	Pop. 05	GA2004230	NAU3731b	*Gt*	-0.93	-	-	0.0211		0.06
Upper Half	Pop. 09	R01-40-08	NAU1014c	*Gh/Gt*	-0.02	-0.03	-2.33	0.0105		0.08
Mean Length	Pop. 12	Paymaster HS26	NAU2432c	*Gh/Gt*	0.02	-0.02	1.00	0.0042		0.09
(UHM)	Pop. 09	R01-40-08	JESPR56c	*Gh/Gb*	-0.03			0.0389		0.05
	Pop. 04	GA2004089	NAU3074c	*Gh/Gt*	-0.03	0.00	0.20	0.0056		0.11
	Pop. 05	GA2004230	NAU3074c	*Gh/Gt*	-0.02	0.00	0.00	0.0137		0.09
	Pop. 04	GA2004089	NAU3731b	*Gt*	-0.04	-	-	0.0044		0.10
	Pop. 05	GA2004230	NAU3731b	*Gt*	-0.03	-	-	0.0207		0.06
Fiber Length	Pop. 02	Paymaster HS26	NAU2998c	*Gh/Gt*	-0.48	-0.91	-1.89	0.0288		0.10
Uniformity Index	Pop. 03	GA2004230	NAU1014c	*Gh/Gt*	-0.70	0.77	1.10	0.0120		0.12
(UI)	Pop. 05	GA2004230	NAU3074c	*Gh/Gt*	-0.54	-0.19	-0.35	0.0032		0.12
	Pop. 05	GA2004230	NAU3731b	*Gt*	-0.56	-	-	0.0447		0.04
	Pop. 08	R01-40-01	JESPR56c	*Gh/Gb*	0.82	-	-	0.0083		0.08
	Pop. 12	Paymaster HS26	NAU2432c	*Gh/Gt*	0.36	-0.28	0.78	0.0319	0.0123	0.09
			NAU5027c	*Gh/Gt*	0.02	-0.55	36.33	0.0449		
	Pop. 17	GA2004089	NAU2432c	*Gh/Gt*	0.57	0.04	-0.07	0.0149		0.11
Short	Pop. 09	R01-40-08	NAU2432c	*Gh/Gt*	-0.09	0.45	-5.00	0.0214		0.06
Fiber Content	Pop. 11	GA2004230	NAU2432c	*Gh/Gt*	-0.45	-0.63	1.40	0.0044		0.18
(SFC%)	Pop. 13	GA2004230	NAU2432c	*Gh/Gt*	-0.50	-0.43	0.86	0.0204		0.11
	Pop. 02	Paymaster HS26	NAU3731c	*Gh/Gt*	-0.44	0.44	-1.00	0.0339		0.09

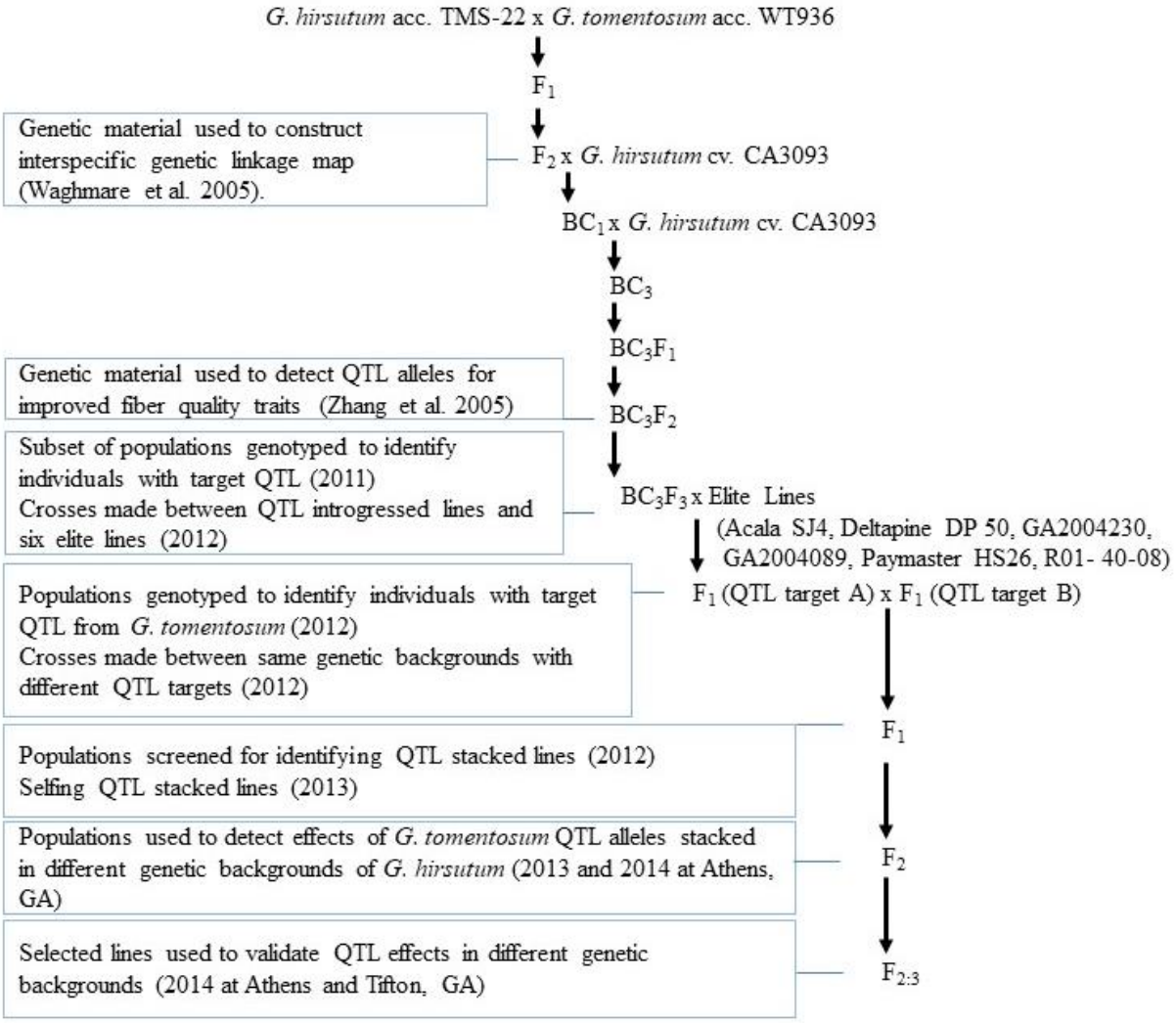
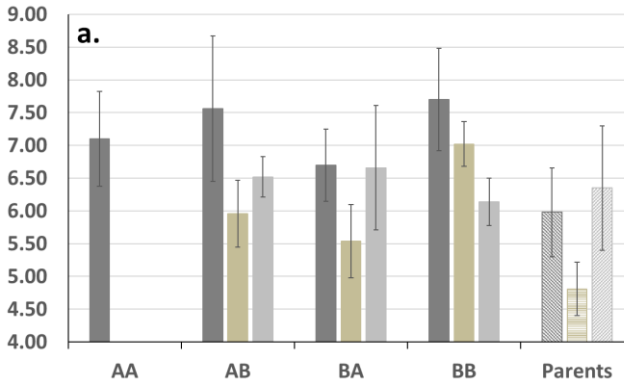


Figure 6.1 Development of populations with *Gossypium tomentosum* and *G. barbadense* QTL alleles stacked in *G. hirsutum* backgrounds.

Figure 6.2 Mean trait values of digenic genotypes (NAU2432-NAU5027) in pooled analysis of selectively advanced $F_2/F_{2:3}$ lines from five QTL-stacked populations. Three *right bars* (Parents) in each figure correspond to R01-40-08, Acala SJ4, and Paymaster HS 26, respectively. Figure 6.3a-6.3f shows average fiber phenotypes [6.3a: fiber elongation (ELO); 6.3b: fiber strength (STR); 6.3c: fiber length (UHM); 6.3d: fiber length uniformity (UI); 6.3e: fiber fineness (MIC); 6.3f: short fiber content (SFC%)] of four, three, and three digenic genotypes (NAU2432-NAU5027) of selected $F_2/F_{2:3}$ lines from pop. 08, pop. 10 and pop. 12 in R01-40-08, Acala SJ4, and Paymaster HS 26 backgrounds, respectively. 'A' refers to homozygous *Gh*-alleles at a locus, 'B' refers to homozygous *Gt*-alleles (first character), and 'H' refers to heterozygous. Error bars show standard deviations.


a.
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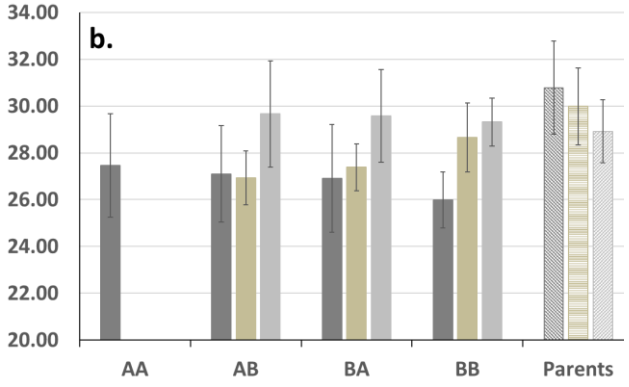
Parents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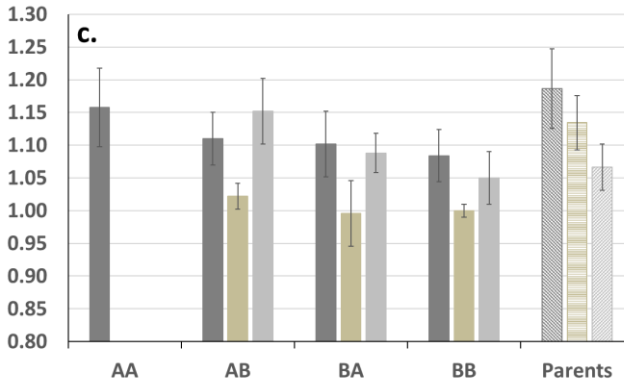
BB
Parents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1.20
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1.10
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1.00
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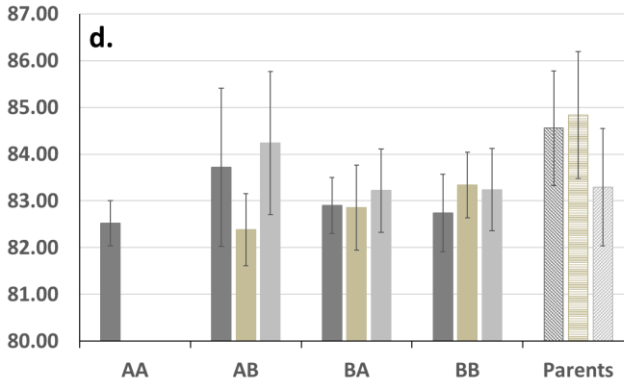
0.90
0.85
0.80
AA
AB
BA
BB
Parents


d.
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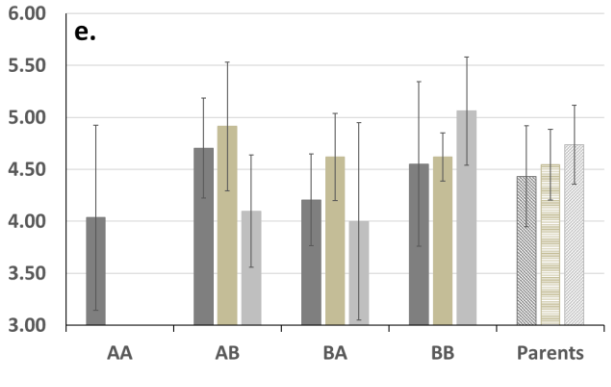
85.00
84.00
83.00
82.00
81.00
80.00
AA
AB
BA
BB
Parents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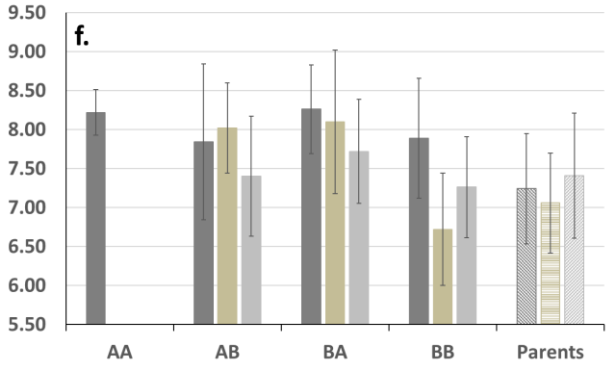
5.50
5.00
4.50
4.00
3.50
3.00
AA
AB
BA
BB
Parents


f.
9.50
9.00
8.50
8.00
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7.00
6.50
6.00
5.50
AA
AB
BA
BB
Parents

CHAPTER 7

SUMMARY

Advances in modern genomics have allowed application of DNA marker technologies to detect and characterize QTL underlying complex traits of interest. In the current study, we used microsatellite markers to study bermudagrass and cotton genetics. In bermudagrass, around 2,000 sugarcane EST-SSR markers were assessed for their cross-transferability and a subset was eventually characterized against a panel of accessions and varieties of interest to turf industry. As the paucity of polymorphic markers in bermudagrass has hindered downstream applications such as rapid and robust varietal characterization, high-resolution linkage mapping, and QTL analysis, reproducible and polymorphic SSR reported in this study are a useful addition. We used these SSRs to substantially enrich a framework linkage map of the bermudagrass genome based on an interspecific cross (*C. dactylon* x *transvaalensis*), providing new insight into chloridoid transmission and evolutionary genetics. We also produced empirical evidence that common bermudagrass exhibited disomic inheritance with irrefutable hints of residual polysomic inheritance, indicating that its current genome constitution is a segmental allopolyploid. Further, we dissected the genetic basis of complex morphological traits of interest in turf bermudagrass. Our early QTL study in bermudagrass adds to molecular breeding resources and information in the genus. There is much potential for advancing current research by increasing the size of the mapping population, increasing marker densities using state-of-the-art genomic tools, integrating parental linkage maps, and collecting more extensive phenotype data (i.e., multi-environment across several locations over multiple years). Turfgrass performance (i.e., establishment and recovery speed, canopy density and uniformity after mowing, and other aesthetic properties) also varies with different management strategies (i.e., mowing height, mowing frequency, fertilizer input, growth-regulator application) and different environmental conditions. Therefore, QTL studies under different management plans and/or growth environments are also warranted. Nevertheless, basic and applied genetic

research in bermudagrass is expected to broaden understanding of evolutionary, structural, and functional genomics of plants.

In cotton, we performed marker-assisted selection for several QTL underlying fiber quality traits and developed 38 early generation QTL-stacked F_2 populations, studied marker-trait association for six standard fiber quality parameters, and assessed QTL-stacked $F_{2:3}$ selections in two different environments (Athens and Tifton GA). The present study demonstrates the practical use of QTL-MAS for transferring fiber quality QTL to *G. hirsutum* cultivars and validating their effects in early generation segregants. Two QTL each from *G. tomentosum* (qFF07.1 and qFE14.1) and *G. mustelinum* (qELO-1-1 and qELO-11-1) were found to increase fiber elongation in different Upland cotton populations and/or backgrounds. The development and characterization of phenotypically superior fiber quality lines in different genetic backgrounds of Upland cotton and validation of several QTL and QTL-stacks represent valuable resource for marker-assisted breeding in cultivar development.