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# A genomic resource for the development, improvement, and exploitation of sorghum for bioenergy

**Zachary W. Brenton**[*,1], **Elizabeth A. Cooper**[*,†], **Mathew T. Myers**[*], **Richard E. Boyles**[*,†], **Nadia Shakoor**[‡], **Kelsey J. Zielinski**[*], **Bradley L. Rauh**[*], **William C. Bridges**[*,§], **Geoffrey P. Morris**[**] **and Stephen Kresovich**[*,†,1]

[*]Institute of Translational Genomics, Clemson University, Clemson, South Carolina 29634, [†]Department of Genetics and Biochemistry, Clemson University, Clemson, South Carolina 29634, [‡]Donald Danforth Plant Science Center, St. Louis, Missouri 63132, [§]Department of Mathematical Sciences, Clemson University, Clemson, South Carolina 29634, [**]Department of Agronomy, Kansas State University, Manhattan, Kansas 66506

**ABSTRACT** With high productivity and stress tolerance, numerous grass genera of the Andropogoneae have emerged as candidates for bioenergy production. To optimize these candidates, research examining the genetic architecture of yield, carbon partitioning, and composition is required to advance breeding objectives. Significant progress has been made developing genetic and genomic resources for Andropogoneae and advances in comparative and computational genomics have enabled research examining the genetic basis of photosynthesis, carbon partitioning, composition, and sink strength. To provide a pivotal resource aimed at developing a comparative understanding of key bioenergy traits in the Andropogoneae, we have established and characterized an association panel of 390 racially, geographically, and phenotypically diverse *Sorghum bicolor* accessions with 232,303 genetic markers. *Sorghum bicolor* was selected because of its genomic simplicity, phenotypic diversity, significant genomic tools, and its agricultural productivity and resilience. We demonstrate the value of sorghum as a functional model for candidate gene discovery for bioenergy Andropogoneae by performing genome-wide association analysis for two contrasting phenotypes representing key components of structural and non-structural carbohydrates. We identified potential genes, including a cellulase enzyme and a vacuolar transporter, associated with increased nonstructural carbohydrates that could lead to bioenergy sorghum improvement. Although our analysis identified genes with potentially clear functions, other candidates did not have assigned functions, suggesting novel molecular mechanisms for carbon partitioning traits. These results, combined with our characterization of phenotypic and genetic diversity and the public accessibility of each accession and genomic data, demonstrate the value of this resource and provide a foundation for future improvement of sorghum and related grasses for bioenergy production.

**KEYWORDS** Bioenergy Association Panel; carbon partitioning; biomass composition; nonstructural carbohydrates

Although numerous plant species have been evaluated as potential bioenergy feedstocks, many of the most promising candidates belong to a tribe of grasses, the Andropogoneae, that includes many agriculturally important species, such as maize, sorghum, and sugarcane. The genetic improvement of bioenergy candidates within this tribe is challenging because little is understood about the genetic architecture of many of their most relevant bioenergy traits. Further complicating this improvement, the Andropogoneae have distinct phenotypic characteristics, such as a Type II cell wall (Vogel 2008), $C_4$ photosynthetic mechanisms, and various carbon partitioning patterns (Braun and Slewinski 2009), which limit the pertinence of basic research in $C_3$ non-grass model organisms, e.g. *Arabidopsis*. Additionally, many of the proposed candidates, such as switchgrass (*Panicum virgatum*) and members of the *Saccharum* genus, including sugarcane, have complex genomes, which limit the generation and


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[1]Institute of Translational Genomics, Clemson University, 317 Biosystems Research Complex, 105 Collings Street, Clemson, South Carolina 29634. E-mail: zwbrent@clemson.edu, skresov@clemson.edu

dissemination of genetic and genomic resources. The designation of a functional model grass species and the subsequent development of a community resource for the genetic dissection of carbon partitioning, biomass composition, and other yield-related bioenergy traits is needed to increase collaboration and accelerate bioenergy improvement.

*Sorghum bicolor* (L.) Moench has emerged as one of the preferred candidates for bioenergy feedstocks and has warranted continued investment for development as a dedicated bioenergy crop due to its high productivity, widespread adaptability, and relative ease of genomic analysis (TERRA 2015). Sorghum is a drought-tolerant, $C_4$ grass with a diverse gene pool that can be exploited for a variety of traits, including those most desirable for bioenergy production. Currently, the existing sorghum germplasm contains four predominant types: grain, sweet, forage, and biomass. Each of which has a preferred ideotype with varying proportions of grain, stalks, leaves, nonstructural sugars, etc. This range of phenotypic diversity not only allows sorghum to serve as a functional model to study various bioenergy and biomass-related traits in Andropogoneae, but it also allows sorghum to be optimized to serve as raw material for promising conversion technologies (Calviño and Messing 2012).

Currently, grain, sweet, and biomass sorghums all serve as feedstocks for various conversion technologies. Grain sorghum, which accumulates starch in the seed, is used as a key feedstock for starch-ethanol conversion throughout the United States (Wu *et al.* 2010). Sweet and biomass sorghums, which are respectively characterized by the accumulation of non-structural and structural carbohydrates in the stalk, provide promise for high-yielding, sustainable bioenergy production. Biomass sorghums have recorded yields of up to 30 dry tons per hectare while sweet sorghums have shown the potential to produce 6,000 liters of ethanol per hectare (Wu *et al.* 2010). Both sweet and cellulosic types have great potential for various bioenergy production methods already in use across locations worldwide. Understanding the genetic mechanisms underlying their differences will be key to maximizing their potential as bioenergy crops.

The distinguishing factor among the different sorghum bioenergy types, and the other bioenergy candidates in general, is how each partitions, translocates, and stores carbon, although the biochemical pathways, machinery, and their genetic controls that allocate carbon to various compositional constituents (i.e., lignin, cellulose, and hemicellulose) are not fully understood (Vogel 2008). Structural carbohydrates, including cellulose, hemicellulose, and pectin, along with the phenolic polymer lignin, are the major components of cell walls (Vogel 2008), while the primary constituent of non-structural carbohydrates in sorghum is sucrose, fructose, glucose, and starch are also present (Saballos 2008). While variation within the structural carbohydrate profile has been documented in sorghum (Murray *et al.* 2008a), few studies have examined the genetic architecture and control of these traits in sorghum or other grasses.

Association studies in sorghum have revealed genetic controls of many phenotypes including height (Brown *et al.* 2008; Murray *et al.* 2009), flowering time (Mace *et al.* 2013a), panicle architecture (Brown *et al.* 2006), seed size (Zhang *et al.* 2015), and various domestication traits (Morris *et al.* 2013a). Most of the studies have been conducted to elucidate the genetic architecture of complex traits as they relate to grain production, not bioenergy production. Because breeding for bioenergy crops with high biomass or fermentable sugars requires a conceptual adjustment from the traditional dwarfed cereals (Salas Fernandez *et al.* 2009), a characterized resource specifically arranged to represent critical bioenergy phenotypes not only allows for greater progress in the explication and exploitation of sorghum's natural genetic diversity, but also the diversity of the broader Andropogoneae tribe.

To facilitate the use of genomic research for improved renewable energy through enhanced biomass-related traits, we created a focused genomic resource, the sorghum bioenergy association panel (BAP). With a total of 390 accessions and 232,303 SNPs, the BAP captures sufficient diversity, yet restricts the panel to bioenergy types to allow for more efficient and informative association mapping. In this study, we introduce the BAP and demonstrate its useful diversity for understanding key bioenergy phenotypes. We also examine the relationship of carbon partitioning between structural, represented by neutral detergent fiber (NDF), and nonstructural carbohydrates, represented by nonfibrous carbohydrates (NFC). Because these traits of carbon allocation are defining characteristics between sweet and biomass sorghum, understanding the genetic controls allows for more efficient improvement by enabling marker-assisted breeding and genomic selection for both types of bioenergy sorghum. Our goal in this research was not only to identify candidate genes that may be the future targets of crop improvement, but also to lay a broader foundation of genetic and genomic resources for future studies that seek to maximize the potential of sorghum and other Andropogoneae as bioenergy crops.

## Materials and Methods

### *Selection and representation of genetic resources*

To ensure the accuracy and availability of this panel for future research, all of the accessions have PI inventory numbers and may be requested through the USDA Germplasm Repository Information Network (GRIN)(File S1). This panel can be divided into two subsets: sweet and biomass types (File S1), which represent to the two most important bioenergy types. Sweet lines were defined as having a Brix value over 10% at the milk development stage or at physiological maturity. The sweet lines consist of 152 accessions, and the 238 biomass types make up the remaining accessions. Sweet accessions include cultivars from previously defined panels: the sweet sorghum association panel (Murray *et al.* 2009) and the U.S. historic sweet sorghum panel (Wang *et al.* 2009). The additional sweet accessions and the biomass lines were chosen based on diversity of worldwide geographic distribution, racial categorization, and agronomic characteristics (File S1). The 390 lines comprise accessions from all five major sorghum races (bicolor, caudatum, durra, guinea, and kafir) with representatives from the entire African continent, Asia, and the Americas (File S1). Several important lines were also added, including lines sequenced at the Joint Genome Institute and the first source of the reference genome, BTx623 (Paterson *et al.* 2009).

### *Field design, phenotypes, and phenotyping protocols*

The BAP was phenotyped in Florence, SC at the Clemson University Pee Dee Research and Education Center in 2013 and 2014. Trials were planted on 76 cm rows at a planting density of approximately 96,000 plants ha$^{-1}$ in loamy-sand soil on 16 May 2013 and 6 May 2014, and were irrigated at the time of planting and on an as-needed basis. Two complete randomized blocks or replicates of the BAP were planted in each year. Due to the extreme height of many of the accessions which were taller than the irrigation pivot, no irrigation took place approximately 90

days after planting. Seed obtained through GRIN (www.ars-grin.gov) was treated with a chemical slurry of Concep II, NipIt, Apron XL, and Maxim XL. This seed treatment allowed for the application of Bicep II Magnum for weed control at a rate of 3.5 L $ha^{-1}$ prior to seed germination. Atrazine at a rate of 4.7 L $ha^{-1}$ was applied before plants had reached a height of 45 cm. Additionally, 125 kg $ha^{-1}$ of layby N was applied approximately 30 days after planting. Besides the chemicals used as part of the seed treatment, no other insecticides or fungicides were applied.

Anthesis was determined when 50% of the plot had begun to shed pollen. Height measurements were taken at physiological maturity, or a set harvest date of Oct 1, from base of the stalk to the apex of the panicle, or if no panicle was present, to the apex of the shoot apical meristem. When possible each plot was harvested at physiological maturity of the genotype with the exception of genotypes that did not flower, which were harvested as a single time point. At the time of harvest, three plants were cut at the base of the stalk, panicles were removed, and fresh weights were recorded. To remove the confounding effects of tillering on a per area basis, yield and compositional data were generated using three representative plants. Based on planting density, this represents approximately 0.5 m of row length. Biomass samples were dried at 40 °C. Dry weight was recorded once samples had obtained a constant weight. Dry tons $ha^{-1}$ was extrapolated based on the dry weight of the samples at the approximate planting density of 96,000 plants $ha^{-1}$. Compositional data, which includes NDF, NFC, Acid Detergent Fiber (ADF), and lignin, were generated by analyzing the dried samples with a Perten DA7250 near-infrared spectroscopy (NIR) instrument (https://www.perten.com). The custom NIR curves were developed by the Perten Applications team using wet chemistry data from 107 unique samples and 10 blind technical replicates generated by Dairyland Labs (www.dairylandlabs.com). Lignin and ADF (a cumulative measurement of lignin and cellulose) wet chemistry data were generated using the Association of Official Agricultural Chemists (AOAC) protocol 973.18 whereas NDF (a cumulative measurement of cellulose, hemicellulose and lignin) and NFC (cumulative measure of nonstructural carbohydrates) data were generated using AOAC protocol 2002.04.The wet chemistry samples were selected based on phenotypic and spectra diversity, a protocol recommended from the Perten Applications team. Yield and composition were compared in the BAP to the Sorghum Association Panel (SAP) (Casa *et al.* 2008), a previously defined sorghum panel focusing on grain sorghum. Dry weights and compositional components in the SAP were calculated based on five representative plants at a rate of 131,000 plants $ha^{-1}$. Compositional data for the SAP were generated using a NIR analysis provided by Chromatin, Inc. (https://www.chromatininc.com). All compositional data are presented as a percentage of dry matter (DM). The USDA-GRIN provided racial and geographic origin information. To provide a control phenotype as confirmation of the genomic data, pericarp pigmentation, which is conditioned by a known gene (Ibraheem *et al.* 2010), was characterized from the seed provided by GRIN following previously outlined methods (Rooney 2000). Phenotypes for the BAP are located in File S2.

#### *Genotyping, SNP calling, filtering, and imputation*

For each entry, five seeds from each plant were grown for two weeks in a growth chamber, and DNA was extracted from whole seedlings using a DNeasy Plant Mini kit from Qiagen. Genotyping-By-Sequencing (GBS) libraries were generated using an ApeKI digestion, and following previously outlined protocols (Elshire *et al.* 2011). Sequencing was performed on an Illumina HiSeq 2000, with 95 barcoded individuals and one negative control included in each lane. Single-end reads for the 343 individuals have been deposited in NCBI SRA under the BioProject identification number PRJNA298892.

Raw sequencing reads were filtered and processed using the TASSEL 5.0 pipeline (Bradbury *et al.* 2007), and BWA (Li and Durbin 2009) was used to align the filtered sequences to sorghum reference genome version 2 available from Phytozome (Goodstein *et al.* 2012; Paterson *et al.* 2009). A minimum aligned read depth of 10 was required for calling SNPs in any individual. (See File S3 and File S4 for details, sample command lines, and Perl scripts.)

After trimming and filtering raw data for quality, we retained over 350 million 64 bp sequencing reads, which corresponded to 1.8 million unique, mapped tag locations in the sorghum genome, and 327,121 putative SNP sites. After filtering low coverage SNPs, individuals with too many missing sites, and sites with a minor allele frequency below 5%, 232,303 SNPs in 343 accessions were retained. Missing genotypes were fully imputed with the software fastPHASE (Scheet and Stephens 2006), with 20 independent starts of the EM algorithm. There is a mean distance of 2-3 kb between each SNP, which is consistent with the level and density of SNP discovery in the previously published SAP (Morris *et al.* 2013a). The fully imputed data set was used for all association analysis and heritability.

To make comparisons between the BAP and the SAP, raw data from both panels were merged, and then filtered using similar methods. However, for these analyses SNPs were filtered with a minor allele frequency of 1% with coverage of at least 60% of individuals and imputed loci with less than 80% confidence were considered missing. The final analyses of allele frequencies and expected heterozygosity were performed on 187,766 common SNPs between the BAP and the SAP.

#### *Genetic differentiation and population structure*

Levels of genetic differentiation between grain, sweet, and biomass sorghums were calculated using Wright's $F_{ST}$ (Wright 1969). For these estimations, we used non-imputed SNP data, and selected sites with a minimum of 80 individuals per type present, as well as a minimum minor allele frequency of 5%. To determine if mean $F_{ST}$ values were significantly different from zero, permutation tests were performed where individual genotypes (across all polymorphic sites) were randomly permuted into groups of the same size 1000 times, and mean $F_{ST}$ was recalculated to determine a null distribution.

Genomic comparisons between the SAP and the BAP were calculated using R statistical software (Team 2011). Expected heterozygosity was calculated using the R package "pegas" (Paradis 2010). Heterozygosity was calculated on a per SNP basis and in a 20 kb sliding window with a 2 kb overlap. The 20 kb region was chosen based on the established LD in sorghum (Hamblin *et al.* 2004; Mace *et al.* 2013b). To determine significance, permutation tests were performed by randomly assigning individuals into groupings of the same size as the original BAP and SAP for 100 permutations. The difference in heterozygosity between the two panels was re-calculated for each permutation, and p-values were generated by counting the number of permuted values which were equal to or greater than the observed heterozygosity difference. Sites with p-values lower than 0.01 were considered significant.

Population structure was estimated using the program STRUCTURE (Pritchard *et al.* 2000). The genetic data were thinned to 1 SNP every 20 kb using the vcftools v0.1.13 thin function (Danecek *et al.* 2011). This left approximately 1 SNP per linkage group. Final structure analysis was performed with 16,476 loci from the 343 individuals with genomic data. Analysis was performed with K values ranging from 1-12. Five independent replicates were generated for each K value with a 10,000 run burnin period followed by 200,000 sampling iterations. Principal component Analysis was conducted using EIGENSTRAT method (Patterson *et al.* 2006) version 6.0.1 using the 'Smart PCA' perl command.

#### *Genome-wide association scans*

Single-SNP tests of association were performed using models implemented in the R package GAPIT (Team 2011; Lipka *et al.* 2012). Association scans were performed using a general linear model (GLM), a mixed linear model (MLM) with internally calculated kinship and population structure, a MLM with kinship and and an externally calculated population structure via STRUCTURE, and the compressed mixed linear model (CMLM) (Zhang *et al.* 2010), which internally controls for population structure and kinship among individuals and uses cluster analysis to assign individuals to groups. The MLMs and the CMLM both incorporate a kinship (K) matrix and population structure (Q matrix), which has been shown to increase statistical power and reduce false positives (Yu *et al.* 2006). Before presenting GWAS results, model fit was compared by examining the QQ plots (File S5), and the CMLM was selected as the model with superior fit. To further reduce the chance of false positives, significance levels in these tests were determined using the Bonferroni correction method resulting in a significance cutoff of approximately 3.0 x $10^{-7}$. Due to an earlier than expected frost in 2013, only 211 were included for genomic analysis. In 2014, a total of 331 individuals were used in genomic analysis.

Linkage disequilbrium (LD) was calculated locally within a 1mb region surrounding each significant locus. Within each region, a pairwise LD between each SNP was calculated using the R Package, 'Genetics'. The extent of LD was determined to decay when the $r^2$ value was less than 0.1 (File S6). Genes potentially linked to any significantly associated SNP were identified by scanning the version 2.1 of the *S. bicolor* genome (Goodstein *et al.* 2012). Gene function was determined using the Panther Classification System (Mi *et al.* 2013) and the European Bioinformatic Institute's PFAM identification (Finn *et al.* 2014). Candidate genes were selected based on functional annotations provided by Phytozome, Panther Classification System, and the PFAM database. SNP effects were predicted by the software snpEff (Cingolani *et al.* 2012).

#### *Phenotypic analysis*

Phenotypic analysis was conducted using R statistical software (Team 2011). Maximum, minimum, mean, and standard deviation values for the BAP were calculated using the mean values of both replicates per year. Phenotypic values in the SAP were calculated based on two replicates in 2013. Accessions that did not flower (i.e., photoperiod sensitive accessions) were not included in the anthesis analysis.

Correlations were determined using the phenotypic mean of the two replicates per year. Pearson correlations and the subsequent p-values were calculated using R statistical software with the cor.test() function. Marker-based estimation of narrow-sense heritability was calculated with the "heritability" package (Team 2011; Kruijer *et al.* 2015).The phenotypic means for each year were treated as replicates in the input. Since the narrow-sense heritability calculation uses the genomic markers (Kruijer *et al.* 2015), a random subset of 100 individuals with complete datasets (ADF, NDF, NFC, lignin, height and dry weight) from 2013 and 2014 were used in the calculations to avoid discrepancies based on genotypes. The centered relatedness matrix used with the marker-based heritability analysis was generated from GEMMA (Zhou *et al.* 2013).

To ensure that phenotypic values (and therefore genomic associations) were not confounded with the block effect, a model was developed for the phenotypic values that included effects of accession and block. Since the blocks contained up to 400 accessions, there may have been field heterogeneity which impacted the phenotypic values. Using the predicted values from the model above (basically the average of the two observations) hopefully minimized the impact of the field heterogeneity. To ensure that the phenotypic values were not confounded with field heterogeneity, an additional model was developed for the phenotypic values that also included covariates associated with the field effect. For this study the covariates chosen were anthesis and height (see descriptions below). Fortunately these covariates turned out to have almost no relationship (not statistically significant) with the primary phenotypes of interest, and even after adjusting for the covariates, the phenotypic values of the accessions remained essentially unchanged (File S7). File S7 also contains the model used for the analysis and the scatterplots for actual and predicted phenotypic values. Therefore we concluded potential field effects were not creating a systematic bias in the phenotypic data and used the predicted phenotypic value for each accession from the model including block effects in the subsequent association analyses. For the GWAS results, values were standardized by subtracting the mean, dividing by the standard deviation, and then averaging across replicates.

## Results

#### *Genomic diversity and differentiation*

To identify genomic regions differentiated between the SAP and BAP, expected heterozygosity was calculated for individual SNPs and within a 20 kb sliding window with a 2 kb overlap. There were 187,766 common SNPs between the panels. Of these SNPs, 14,841 loci differed in expected heterozygosity by more than 25%. To look at global patterns in differentiation between the two resources, the SNPs were divided into sliding windows of 20 kb representing genomic regions within the estimated LD distance and the mean heterozygosity for each block was compared (Figure 1). This resulted in 26,110 regions in which 525 differed in the expected heterozygosity by more than 25%. Since grain types have been selected for early maturity in temperate environments for grain maturation and bioenergy types have been selected for delayed flowering and increased biomass, it would be expected that regions surrounding major maturity genes would differ in the expected heterozygosity. To test this hypothesis, the expected heterozygosities of the 20kb flanks surrounding known maturity genes ($Ma_1$ (Murphy *et al.* 2011), $Ma_3$ (Childs *et al.* 1997), and $Ma_6$ (Murphy *et al.* 2014)) and a known dwarfing gene ($Dw_3$ (Multani *et al.* 2003)) were compared between the two panels. The regions surrounding $Ma_1$, $Ma_3$, and $Dw_3$ in the BAP and SAP were significantly different whereas $Ma_6$ was not. There was low SNP coverage around the $Ma_6$ locus which may explain why the $Ma_6$ locus was not

differentiated between the two data sets. Although the SAP had a greater average heterozygosity near $Ma_1$, regions surrounding $Ma_3$ and $Dw_3$ had higher average heterozygosities in the BAP than the SAP (Figure 1). This data highlights the fundamental differences in the two panels and suggests that there may be unexploited genetic diversity in the BAP due to a selective bottleneck for dwarfed, early-maturing grain accessions in temperate environments.

Because sweet and biomass sorghum are the primary types used for bioenergy production, determining how differentiated these two types are could provide insights into the genetic architecture of compositional components. However, the level of differentiation (as measured by $F_{ST}$) between the sweet and biomass types of sorghum was overall very low (mean $F_{ST}$ = 0.024, where 0 = no differentiation and 1 = complete differentiation), although it was significantly greater than the null distribution (File S8). The maximum value of $F_{ST}$ is 0.276, highlighting that there were no fixed differences between types in the data set despite significant phenotypic differences.

#### *Population structure*

Previous work in the SAP has shown that population structure is related to the categorization of sorghum to the five botanical races and numerous geographic regions of sorghum colonization (Casa *et al.* 2008; Brown *et al.* 2011). Previous work has also demonstrated that these phenotypically based classifications are genetically supported (Brown *et al.* 2011). Based on these observations it would be expected that similar population patterns would appear in the BAP. Definitive patterns emerged supporting the previous findings that race and geographical origin help define subpopulation categorization (Figure 2). Figure 2 shows the STRUCTURE results from K=6 of 343 individuals in the BAP. As expected, each of the five botanical races emerge as subpopulations. Additionally, a sixth cluster appears which divides the Ethiopian accessions into two distinct groups. Since Ethiopia is the center of diversity for sorghum, it is not unexpected that distinct subpopulations could emerge when analyzing population structure. Racial data was not provided by GRIN for any of the accessions included in the orange cluster (Figure 2). Since racial classification is determined, at least in part, by panicle architecture and seed characteristics, it was not possible to establish racial classifications for this group due to the limited panicle emergence in the photoperiod sensitive accessions. Interestingly, the most distinct group, the guinea population in the green cluster, cluster heavily together and have the lowest proportion of membership. Principal Component Analysis also showed clustering of the West African guinea types as well as the unclassified Ethiopian accessions. Additional STRUCTURE and Principal Component Analysis results are in File S9.

#### *Phenotypic means, distributions, correlations and heritability*

To highlight the differences between the grain-dominated SAP and the BAP, data were collected for phenotypes important for bioenergy sorghums. Comparison between the two panels revealed distinct patterns of phenotypic selection for each of the two types (Table 1). The average anthesis date in the BAP was almost 30 days longer than the SAP. This would have been even greater if photoperiod sensitive lines were included in the analysis. The average height was nearly two meters greater in the bioenergy than the grain panel. Also, the accumulation of above ground biomass was significantly greater in the bioenergy panel. The composition traits as a proportion of dry matter (DM) did not differ as much between the two panels; however, when extrapolating the compositional components based on the dry weight, differences between two panels become more apparent. For example, the average accumulation of NDF ha$^{-1}$ would be nearly 13 tons versus 6 tons in the SAP. Not surprisingly, NFC as a percentage of DM is higher in the BAP than the SAP. Since 139 of the accessions in the BAP are classified as sweet types that have been selected to accumulate nonstructural carbohydrates, it is reasonable to expect that the BAP would have a higher percentage and maximum value for the accumulation of nonstructural sugars.

Of the phenotypes collected in the BAP, the marker assisted narrow-sense heritability estimates were generally high. Overall, the heritability of each phenotype is similar to previously published work (Table 2). However, anthesis heritability was much higher in the BAP than previously published studies (Murray *et al.* 2008a). This may be because many of the accessions in the BAP rely on photoperiod induction to initiate reproductive tissue formation. Since the heritability estimation used data from only one geographic location, the heritability estimate likely does not reflect the actual impact of the various latitudes and day lengths on photoperiod sensitive lines. If anthesis values were collected in an environment with a shorter day length and the same analysis was conducted to calculate heritability, these values would probably be much lower. The compositional phenotype heritabilities were similar to previously published results (Murray *et al.* 2008a).

#### *Validation of GWAS using seed color as a control*

Pericarp pigmentation in sorghum seeds is a well-studied trait that is known to be controlled by a MYB transcription factor (*Y1*; *Yellow seed1*) (Rooney 2000; Ibraheem *et al.* 2010; Morris *et al.* 2013b). Since this gene has been mapped in the SAP (Morris *et al.* 2013b), pericarp pigmentation was used as a control in this study to validate the genetic data. As expected, all of the models in GAPIT (GLM, MLM, and CMLM to control for population structure and kinship) identified a single region within the transcript of the *Y1* locus (*Sobic.001G397900*) that was strongly associated with seed color in the BAP (Figure 3).

#### *Association mapping for structural and nonstructural carbohydrates*

Association mapping revealed genomic regions strongly associated with NDF and NFC. Since these phenotypes are inversely related to one another, it would be expected that many of the same significant loci identified for one phenotype were also present in the other phenotype. The association scans from NDF and NFC demonstrate this relationship (Figure 4).

Using the CMLM from GAPIT, the association scans revealed a total of 8 significant SNPs representing 5 loci and 22 genes (File S10). LD was calculated locally for each significant SNP (File S6). Significantly-associated SNPs within the distance of LD decay of on another were considered a single locus; also, any gene within the LD estimate was considered linked, and plausibly implicated in the determination of the phenotype. Of the 8 significant SNPs, two are intragenic missense variants, indicating the higher likelihood that specific genes contribute to the phenotype.

A total of five regions were identified through the association methods: two loci were located on Chromosome 4 and three on Chromosome 6. Although most loci identified had plausible explanations of their impact on biomass compositional

components, one of the regions on Chromsome 4 is particularly interesting. A SNP in this region causes an amino acid change to a vacuolar iron transporter. The SNPs in this linkage group appear to create a distinctive haplotype structure. There were three haplotypes in this region (Figure 5). The mean NFC of haplotype III was 41.8% while the mean value of haplotype I was only 25.5% NFC. Haplotype II, which only differed from haplotype I by a single base pair, also had a low NFC value (21.5%). Of the individuals with NFC over 40% DM (29 individuals), 11 individuals have haplotype III. The top five individuals all have haplotype III at this location. Historically important sweet lines such as *Rio*, *Wray*, *Leoti*, and *Sugar Drip* each possessed haplotype III at the specified locus (Figure 5). The strong association with NFC coupled with the clustering of historically important accessions provides evidence that this region impacts the accumulation of nonstructural carbohydrates in *Sorghum bicolor*, and could be important for bioenergy sorghum improvement.

Due to the potentially confounding effects of height and maturity on accumulation of structural and nonstructural carbohydrates, the candidate genes were compared to the locations of known maturity genes ($Ma_1$-$Ma_6$) (Mace and Jordan 2010) and known dwarfing genes ($Dw_1$-$Dw_4$) (Mace and Jordan 2010) . There was no co-localization among any of the maturity genes or dwarfing genes with any of the significantly associated regions. Furthermore, there was no overlap among the nearly 221 candidate genes identified for maturity (Mace *et al.* 2013a) and the candidate genes for structural or nonstructural carbohydrates identified in this study. In addition, GWAS were conducted on height and flowering time from the data in the BAP; no significant SNPs co-localized with the results from NFC and NDF (File S11).

### *Candidate gene identification*

Each region identified in through GWAS has plausible candidates for biomass composition (Table 3). Most notably, SNP S4_63347613, shown in Haplotype III (Figure 5), causes an amino acid change from an alanine to a valine in a vacuolar iron transporter family protein. Previous studies have shown that sucrose accumulation in plants regulates an iron-deficient response (Lin *et al.* 2016). Furthermore, in a previous comparison of divergence between sweet and grain types, this region underwent a segmental duplication from their most recent common ancestor, suggesting possible neofunctionalization of the two VIT between sweet and grain sorghum (Jiang *et al.* 2013). Additionally, a vacuolar-processing enzyme was identified in this region. Vacuoles serve a major role in sucrose accumulation and mobilization in plants (Leigh 1984). The other region on Chromosome 4 contains four genes. One of which, a B-box zinc finger protein, shares homology with a salt tolerance homolog. Sugar accumulation has been shown to be a molecular response to salt stress in sorghum (Sui *et al.* 2015).

The region identified on Chromosome 6 had two genes coding for Cellulase enzymes, *Sobic.006G122200* and *Sobic.006G122300*. These genes hydrolyze glycosidic bonds in complex carbohydrates, such as cellulose, which is the major component of NDF. These SNPs were associated with increased levels of nonstructural carbohydrates and decreased levels of structural carbohydrates. These glycoside hydrolase family 5 proteins could be involved with the degradation of structural components of the cell wall. These were the only two genes to have GO terms associated with carbohydrate metabolic process (GO:0005975). Additionally, a Transducin/WD40 family protein was identified from a significantly associated SNP 773 bp upstream. Transducin/WD40 proteins have been shown to increase biomass accumulation (Gachomo *et al.* 2014). Although the genes identified in this study are plausible candidates for biomass compositional components, further evidence will be needed to dissect the true effect of these allelic variants.

## Discussion

### *Sorghum as a functional model for bioenergy and the value of the BAP*

Of the potential bioenergy Andropogoneae candidates, sorghum has emerged as one of the preferred species for direct commercialization as a bioenergy crop and as a functional model for other Andropogoneae. Sorghum has natural advantages as a model for this family of grasses because of its relatively small diploid genome (~ 730 Mb), significant breeding history, and substantial natural diversity. This extensive genetic and phenotypic diversity provides the foundation for gene discovery and crop improvement. It also allows sorghum to serve as a model for other bioenergy Andropogoneae because of its adaptability to various bioenergy conversion technologies. Due to its high levels of sugar accumulation and its close evolutionary history, it can also serve as a relevant reference for the *Saccharum* genus. Since there are no reported genomic incompatibilities among the four types of sorghum, genes identified that improve bioenergy sorghum performance in the BAP could be incorporated into grain and forage types as well.

The BAP was constructed by using publicly available racial and geographic as well as agronomic data from field evaluations. Since previous studies have shown that the racial classifications are genetically supported (Brown *et al.* 2011), the hypothesis was that by selecting lines incorporating the major botanical races, we would be able to capture a sufficient amount of genetic diversity. The botanical races are correlated with geographic regions. After we selected individuals based on racial distribution, we supplemented under represented regions with accessions with known geographic origins. Phenotypically, we restricted accessions to tall photoperiod sensitive, late maturing accessions. We also chose accessions screened for resistance to a major sorghum disease, anthracnose. This was an attempt to remove the confounding effects of varying resistances and susceptibilities since the presence of the disease could alter the carbon composition profile of the individual accession. Although we tightly constrained the amount of diversity for flowering time, height, and disease susceptibility, we capture an appropriate amount of genomic diversity compared to other panels. Finally, historically important lines used in breeding and lines that were sequenced at the Joint Genome Institute were included. All accessions are available for public distribution through the USDA's GRIN system.

The development of a genetic and genomic resource specifically designed to capture the natural genetic and phenotypic diversity of sorghum for carbon partitioning and biomass composition increases the efficiency and efficacy of association genetics and incorporation of favorable alleles into a breeding pipeline. Although nested association mapping populations (NAM) and multi-parent advanced generation inter-cross (MAGIC) populations have been shown to improve the detection of small effect loci and reduce the false-discovery rate (Cavanagh *et al.* 2008; Yu *et al.* 2008), these populations severely restrict the diversity and thus the detection of novel gene candidates or rare, favorable alleles. In addition, diversity panels developed for conservation of

genetic resources and analysis of genetic diversity impede many efforts to identify causal genes either because of the confounding effects as a consequence of the diversity or the lack of statistical power from a low phenotypic frequency. The BAP's construction limits the confounding effects associated with flowering time and height (Flint-Garcia *et al.* 2005) by limiting the panel to tall, late-flowering photoperiod sensitive accessions. Furthermore, the selection of accessions with known phenotypic diversity increases the likelihood that variants are at higher frequencies in the mapping population, which increases the probability of a true positive association (Myles *et al.* 2009). The creation, evaluation and characterization of a diversity panel with the public dissemination of data provides insights to create better constructed NAM, MAGIC, recombinant inbred lines (RILs), or candidates for whole-genome re-sequencing. Overall, the BAP was created to overcome the limitations with other genomic resources, and the effective mapping of two key phenotypes show the advantages of using the BAP for critical bioenergy traits, but future studies should implement better field designs for improved statistical analysis. An important insight from this study is that the large number of accessions allowed a thorough analysis of the associations, but resulted in a design with very large block size. Even though we corrected for possible field heterogeneity from the large block size, additional studies using this resource should utilize superior designs such as an incomplete block design with multiple row plots. This allows for adjustment due to competition effects and other field variants. With more appropriate design, the BAP has the potential to serve as a critical resource for the continued advancement of sorghum as a preferred bioenergy feedstock.

### *Conclusion*

The objective in this study was to expand the existing foundation of genetic and genomic resources for bioenergy research in non-model Andropogoneae. By creating the sorghum BAP, we provide a genetic and genomic resource that not only provides a foundational knowledge for determining the genetic architecture of traits important for bioenergy, but also expands the current germplasm in the sorghum community. Although this panel limited phenotypic variance of the included accessions to bioenergy-like ideotypes, genetic and phenotypic diversity of the overall species was maintained. The strong heritabilites and the low correlations of the compositional phenotypes to dry weight suggested that composition can be improved without affecting the total yield (Murray *et al.* 2008b). The association analysis identified regions of the genome that could be targeted to improve biomass quality. However, others have suggested that increasing total yield is more important than improving composition quality for maximizing extractible energy per unit input (Murray *et al.* 2008a). Since increasing sink strength has been shown to advantageously affect yield (Bihmidine *et al.* 2013), understanding the genetic controls of the compositional components could allow for improved sink strength with a positive yield outcome. By identifying genomic regions independently affecting yield and composition, researchers could simultaneously select for both yield and increased quality instead of selecting for one or the other. This would allow researchers to increase yield and compositional quality concurrently promoting an increase in breeding efficiency and bioenergy optimization. Furthermore, determining the genetic controls of carbon allocation in sorghum may be useful in elucidating the genetic mechanisms controlling biomass yield, sugar accumulation, and other compositional constituents in other $C_4$ grasses.

By analyzing phenotypic and genomic data from the BAP, researchers can better design experiments to study the genetics of bioenergy sorghum. Providing corroborating evidence on how sorghum populations are structured not only reinforces previous studies (Morris *et al.* 2013a; Casa *et al.* 2008), but also provides valuable information pertaining to how certain botanical races of sorghum may perform in a bioenergy context. The establishment, characterization, and subsequential genomic analysis of this resource have highlighted regions of the genome and possible candidate genes for targeted improvement in bioenergy sorghum. These candidate genes need further validation, such as analysis of segregating populations, targeted gene sequencing, and functional tests. The need for the grass community to develop appropriate resources for gene identification with functional annotations is imperative for the continued improvement of bioenergy feedstocks. The creation and analysis of this foundational resource provides researchers with valuable tools and essential knowledge for continued experimentation with bioenergy sorghum and other Andropogoneae. Providing easily accessible accessions with genomic information allows for greater efficiency of research by encouraging collaboration and the dissemination of information. The establishment, characterization, and analysis of the BAP facilitate the advancement of sorghum for bioenergy production and optimization worldwide, and provide a foundational resource for the development of renewable energy.

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**Table 1 Phenotypic comparisons between the SAP and BAP**

| | BAP | | | | | SAP | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Phenotype | N | Average | Min. | Max. | Std. Dev. | N | Average | Min. | Max. | Std. Dev. |
| Anthesis (days) | 217 | 97 | 66 | 153 | 24 | 369 | 68 | 50 | 111 | 7 |
| Height (cm) | 390 | 341.2 | 75.0 | 536.0 | 86.8 | 383 | 147.3 | 63.5 | 414.5 | 57.7 |
| Dry Weight (tons $ha^{-1}$) | 390 | 19.4 | 3.3 | 70.9 | 11.3 | 344 | 7.7 | 2.21 | 28.6 | 3.9 |
| ADF (% of DM) | 387 | 41.5 | 14.0 | 54.9 | 7.9 | 379 | 37.5 | 24.8 | 61.2 | 5.5 |
| NDF (% of DM) | 387 | 67.1 | 47.1 | 81.2 | 7.1 | 379 | 62.9 | 43.2 | 78.4 | 6.1 |
| NFC (% of DM) | 387 | 27.6 | 13.9 | 50.0 | 8.0 | 369 | 20.3 | 10.5 | 45.5 | 6.4 |
| Lignin (% of DM) | 387 | 6.6 | 1.6 | 10.5 | 1.6 | NA | NA | NA | NA | NA |

**Table 2 Heritability and correlations of phenotypes in the BAP**

| Phenotype | $H^2$ Calculation | $h^2$ Estimation | Anthesis | Height | Dry Weight | ADF | NDF | NFC | Lignin |
|---|---|---|---|---|---|---|---|---|---|
| Anthesis | 0.86 | 0.90 | - | 0.724*** | 0.687*** | 0.530*** | 0.163* | -0.088 | 0.579*** |
| Height | 0.72 | 0.82 | 0.724*** | - | 0.549*** | 0.430*** | 0.245*** | -0.141** | 0.527*** |
| Dry Weight | 0.39 | 0.32 | 0.687*** | 0.549*** | - | 0.009 | -0.088 | 0.183*** | 0.056 |
| ADF | 0.55 | 0.62 | 0.530*** | 0.430*** | 0.009 | - | 0.837*** | -0.866*** | 0.872*** |
| NDF | 0.51 | 0.54 | 0.163* | 0.245*** | -0.088 | 0.837*** | - | -0.963*** | 0.721*** |
| NFC | 0.50 | 0.56 | -0.088 | -0.141** | 0.183*** | -0.866*** | -0.963*** | - | -0.704*** |
| Lignin | 0.57 | 0.70 | 0.579*** | 0.527*** | 0.056 | 0.872*** | 0.721*** | -0.704*** | - |

*Signifiance at 0.05 probability; **significance at 0.01; ***significance at 0.001.

**Table 3 Significant SNPs, candidate genes, and number of genes within LD of significant SNP**

| SNP | P-value | Local LD | Num. of genes in region | Candidate gene | Distance to candidate gene (bp) |
|---|---|---|---|---|---|
| S4_63301409 | $6.85 \times 10^{-8}$ | 23kb | 4 | Salt-tolerance homolog | 18,095 downstream |
| S4_63301429 | $6.85 \times 10^{-8}$ | 23kb | 4 | Salt-tolerance homolog | 18,105 downstream |
| S4_63347613 | $1.41 \times 10^{-7}$ | 23kb | 8 | Vacuolar iron transporter | Intragenic |
| S4_63347623 | $1.41 \times 10^{-7}$ | 23kb | 8 | Vacuolar iron transporter | Intragenic |
| S6_4320818 | $4.40 \times 10^{-8}$ | 1kb | 0 | NA | NA |
| S6_4330906 | $1.64 \times 10^{-7}$ | 1kb | 0 | NA | NA |
| S6_49773083 | $1.68 \times 10^{-8}$ | 16kb | 9 | Cellulase (Glycosyl hydrolase) | 13,666 downstream |
| S6_49784457 | $1.48 \times 10^{-8}$ | 16kb | 4 | Transducin/WD40 homolog | 773 upstream |

***Genome-wide heterozygosity patterns in the BAP and SAP with selected maturity and height loci***

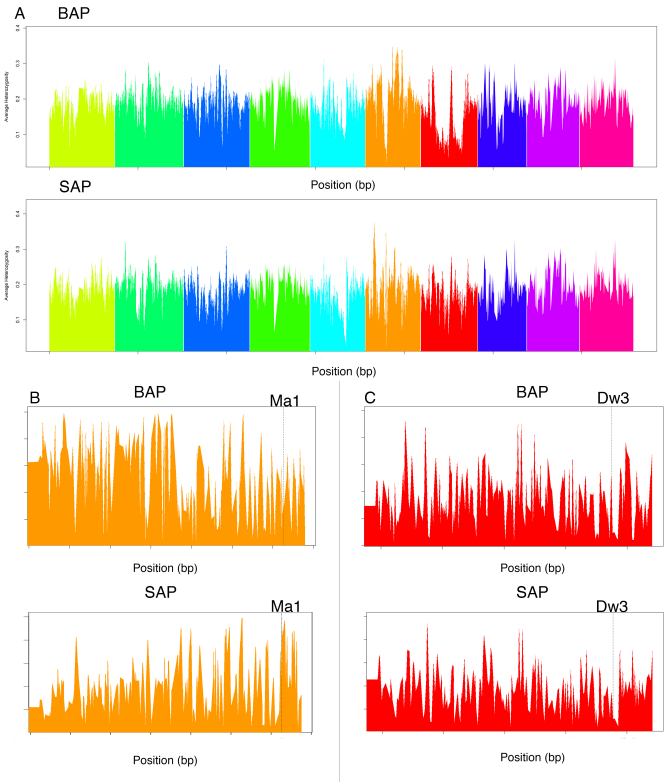


**Figure 1** A) Genome-wide heterozygosity calculated for the BAP (top) and SAP (bottom) with a 500 kb sliding window. B) Average heterozygosity in 20 kb windows with 2 kb overlap for the region on Chromosome 6 containing the $Ma_1$ gene, *Sobic.006G057900*, in the BAP (top) and the SAP (bottom). C) Average heterozygosity in 20 kb windows with 2 kb overlap for the region on Chromosome 7 containing the $Dw_3$ gene, *Sobic.007G047300*, in the BAP (top) and the SAP (bottom).

***Population structure of the BAP***

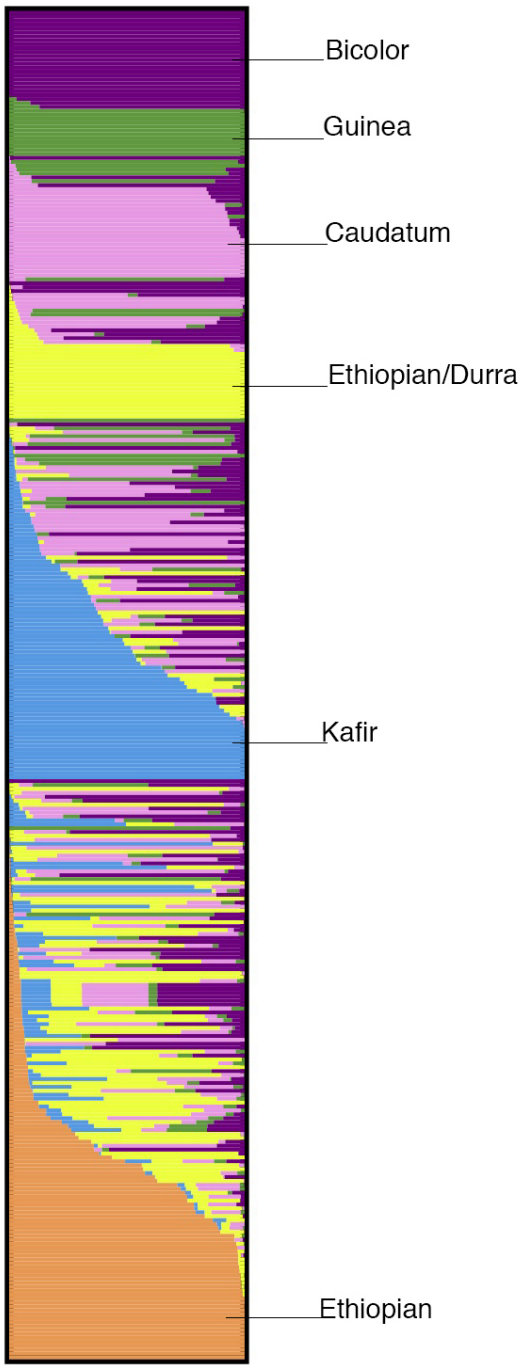


**Figure 2** Population structure results with six defined subpopulations. The purple cluster represents bicolor accessions. The green cluster has the fewest number of members and is mainly made of up guinea accessions. The pink cluster represents Caudatum accessions. The yellow cluster represents Durra accessions that are mainly from Ethiopia. The blue cluster includes individuals that cluster with Kafir types. This grouping is usually associated with photoperiod insensitivity. The orange cluster represents accessions from Ethiopia, but no racial data was available for these lines.

***GWAS results for pericarp pigmentation in the BAP using multiple models***

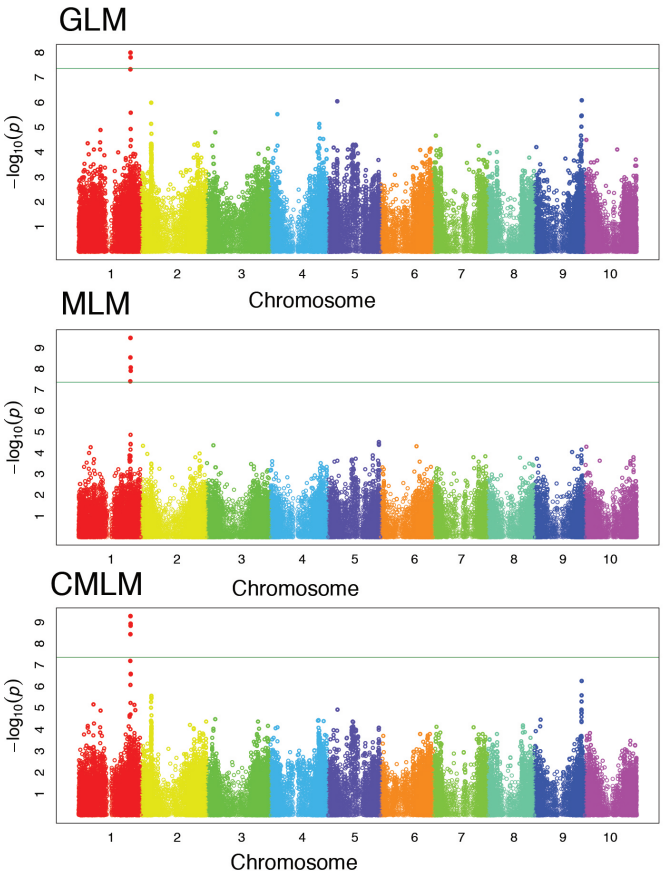


**Figure 3** A single locus, the *Y1* MYB transcription factor, was identified in all three models as expected. This phenotype represents a control to validate correct SNP calling, imputation, and GWAS methodology.

***GWAS results for NFC and NDF***

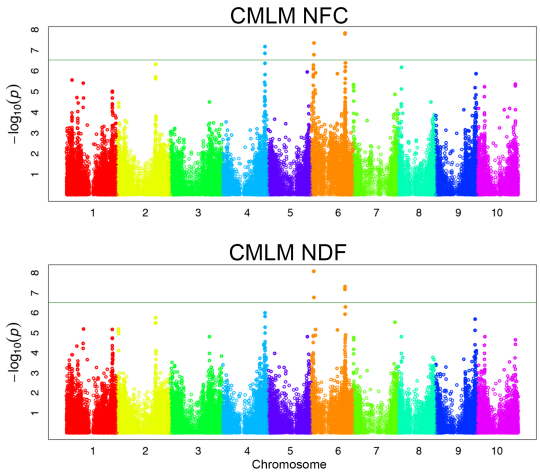


**Figure 4** A total of 8 unique SNPs, 5 loci, and 22 genes were identified using the CMLM for NFC and NDF. SNPs with a p-value less than $3.00 \times 10^{-7}$ were considered significant.

***Haplotypes on Chromosome 4 in the region significantly associated with NFC***

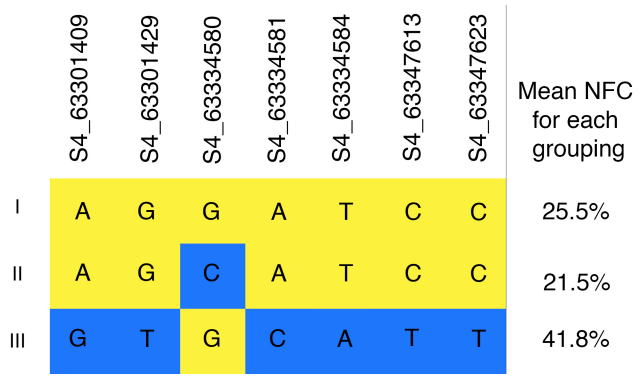


**Figure 5** Three haplotypes on Chromosome 4. This region was significantly associated with NFC in the CMLM in 2014. The yellow indicates the more frequent allele, and the blue indicates the less frequent allele. Haplotypes I and II correspond to low values of NFC while haplotype III corresponds to high levels of NFC.

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