CHARACTERIZATION OF PERENNIAL TRAITS IN HYBRIDS BETWEEN MAIZE AND PERENNIAL TEOSINTE

by

CAROLINE COATNEY

(Under the Direction of R. Kelly Dawe)

ABSTRACT

Genetic mapping and quantitative trait locus/loci (QTL) mapping were used to study the genetic factors associated with perenniality in a cross between the annual maize inbred B73 (*Zea mays* ssp. *mays*) and a perennial teosinte relative, *Zea diploperennis*. The perennial-related traits analyzed were flowering time, tiller number, regrowth after autumn cutback, stay-green, and overwintering. An F_2 population of 482 individuals was phenotyped and a subset of 93 F_2 individuals was genotyped using 858 single nucleotide polymorphism (SNP) markers generated with genotyping-by-sequencing (GBS). A genetic map was developed that totaled 1,437.5 cM in length with an average of 85.8 SNPs per chromosome. Associations between phenotypes and genotypes were analyzed with composite interval mapping. One QTL was detected, which was for the stay-green phenotype on chromosome 5 that was responsible for 27.0% of the phenotypic variation.

INDEX WORDS: Perenniality, *Zea diploperennis*, Genetic map, Genotyping-by-sequencing, Wide hybridization, Stay-green, QTL mapping

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Perennial cereal grain crops

The dichotomy between perennials and annuals is one of many used to divide and organize plant species. In recent years, the potential fluidity between perennial and annual states, and the usefulness it could have in an agricultural context, has been seriously discussed in the scientific community (Cox et al. 2002; DeHaan et al. 2005; Cox et al. 2006; Cox et al. 2010; Glover et al. 2010; Van Tassel & DeHaan 2013; DeHaan & Van Tassel 2014). Specifically, if the genetic factors differentiating perennial plants from annual plants are discovered, annual crops could be converted to perennials and revolutionize farming in parts of the world.

There are many life history traits that distinguish perennials from annuals. Annuals propagate by seeds and have one growing season. Perennials multiply and disperse by both seeds and vegetative organs—such as rhizomes, stolons, tubers—and live for two or more growing seasons. These vegetative organs allow perennial plants to overwinter and quickly regrow at the start of the next growing season. Early leaf canopy production can be a weed deterrent since it shades out weedy competitors. Converting annual crops to perennial crops would not only take advantage of early biomass production, but could also bring to fruition several other benefits. Unlike annual crops, perennial crops could reduce soil erosion and store more nutrients and carbon below

ground because of their large root structure and storage organs, and could also require less water and fertilizer since their deeper roots allow access to deeper resources (Grime 1977; Cox et al. 2002; DeHaan et al. 2005). These advantages make perennials particularly well adapted to thrive on marginal lands (Tilman et al. 2009), where they would be most useful to third world subsistence farmers.

The majority of the world's calories are supplied by cereal grain crops. The major cereal grain crops are rice, wheat, and maize. Since 1998, maize has been the most produced cereal grain crop in the world (FAOSTAT Database, http://faostat.fao.org/). However, it does not have nearly the same prominence in the realm of perennial research. Here, we attempt to contribute information and data to elucidate the potential of converting maize to a perennial crop.

The teosinte taxa

Domesticated maize, *Zea mays* ssp. *mays*, is one of the most recognizable and most researched plant species in the world. This attention, especially from the scientific community, has revealed the intimate details of its biology as well as the biology of the species most closely related to it, including the teosinte taxa. The teosintes are a group of plants native to a range spanning Mexico, Guatemala, and Nicaragua that is composed of ecologically diverse environments in terms of precipitation, elevation, and temperature (Hufford et al. 2012). The members of the teosinte taxa include the perennials *Zea diploperennis* and *Zea perennis* (the only tetraploid teosinte), and the annuals *Zea luxurians*, *Zea nicaraguensis*, and *Zea mays*. The latter is composed of four subspecies:

(1) ssp. *mays*, domesticated maize, (2) ssp. *mexicana*, (3) ssp. *huehuetenangensis*, and (4) ssp. *parviglumis*, the progenitor of maize (Matsuoka et al. 2002).

Of these teosintes, *Z. perennis* and *Z. diploperennis* are of particular interest to our work due to their perennial habit. *Z. perennis*, first discovered in 1922 (Hitchcock 1922), was previously known as *Euchlaena perennis* until it was taxonomically redefined to be included in the *Zea* genus in 1942 (Reeves & Mangelsdorf 1942). It is an autotetraploid (2n = 4x = 40) found in distinct populations on the mountain slopes of Jalisco, Mexico (Hufford et al. 2012). Much effort has gone into studying the cytological relationship and hybridization potential between *Z. perennis* and diploid domesticated maize. Some researchers have focused on the triploid hybrids produced while others have either reduced the *Z. perennis* genome or doubled the domesticated maize genome before crossing (Emerson and Beadle 1930; Molina 1978a; Molina 1978b; Mazoti and Rimieri 1978; Vina and Ramirez 1994; González et al. 2006; Allen 2005; Takahashi et al. 1999; Shaver 1964; Shaver 1962; Emerson 1929; Tang et al. 2005; Shaver 1963). Differences found between parental chromosomes include position of centromeres, length of chromosomes, and the presence or absence of knobs.

The second perennial teosinte, *Z. diploperennis*, is a diploid (2n = 2x = 20). Originally thought extinct in 1921, *Z. diploperennis* was rediscovered in 1979 at two locations on the mountain slopes of southern Jalisco, Mexico (Iltis et al. 1979). Iltis et al. describe it as morphologically distinct from *Z. perennis* by its two different types of rhizomes, less dense root system, increased number and length of tassel branches, wider and longer leaves, and more "robust habit." Since its discovery, it has become the

preferred perennial teosinte for study most likely due to its ploidy compatibility with domesticated maize and the majority of the teosintes.

Perennial research in maize

Before the rediscovery of *Z. diploperennis* in 1979, the few attempts made to develop a perennial maize variety were limited to using *Z. perennis* as the perennial donor. Shaver (1964) made the first attempt to develop perennial maize by trying two different crossing schemes. His first crossing scheme began with crossing *Z. perennis* with diploid maize and then followed by backcrossing F_3 individuals to the maize parent. This resulted in triploid plants that readily grew shoots and roots from culms separated from the main root mass, which Shaver believed were indicative of perenniality. His second crossing scheme used *Z. perennis* and colchicine-induced tetraploid maize to produce tetraploid hybrid progeny. After strongly selecting for tillers for several generations, he found 70% of F_4 individuals had a significant number of tillers, which he equated with perenniality. However, after backcrossing to tetraploid maize, no plants were perennial. For both crossing schemes, plants were overwintered in the greenhouse.

From his triploid work, Shaver was able to produce a 22-chromosome hybrid that showed what he believed to be the perennial phenotype (Shaver 1964). A low-frequency crossover event that allowed the postulated perennial gene, *pe*, to be present in combination with the recessive genes for indeterminacy (*id*) and grassy tillers (*gt*) in a diploid background was thought to be responsible for the observed perennial phenotype (Shaver 1967). All material was grown at winter nurseries in either California or Florida.

However, tiller number was used as the primary metric for the perennial phenotype, which some believe to be a potentially misleading technique (Cox et al. 2002).

In the 1980s, the search for the genetic basis of perenniality in *Zea* shifted away from *Z. perennis* and towards *Z. diploperennis*, after the latter's rediscovery. Mangelsdorf and Dunn (1984) hypothesized in the Maize Genetics Cooperation Newsletter that *Z. diploperennis* was perennial due to its rhizomes, which they believed was a recessive trait, and that an associated gene was located on the long arm of chromosome 4. Later, Srinivasan and Brewbaker (1999) crossed *Z. diploperennis* with eleven Hawaiian tropical maize inbreds. They quantified several morphological traits, including tiller number, in the F_1, F_2, BC_1, and BC_2 populations over the course of three seasons in Hawaii. However, they were not able to produce any perennial plants. Westerbergh and Doebley (2004) took a quantitative trait locus/loci (QTL) mapping approach and crossed *Z. diploperennis* with the annual teosinte *Z. mays* ssp. *parviglumis*. They scored the F_2 population for eight traits associated with branching, withered stems, rhizomes, underground stems and roots, and tillers; however, all plants were dug up for underground characterization at the end of the first growing season, preventing any overwintering analysis. Thirty-eight QTL were found for the eight traits. QTL for six traits mapped near each other on chromosome 2, and QTL for four traits mapped near each other on chromosome 6. Two QTL were identified for rhizomes, which explained less than 12% of the variation. Nine QTL were identified for number of tillers, which explained almost 45% of the variation (Westerbergh & Doebley 2004).

Perennial genetics research in other taxa

Rice

Research on the genetics of perenniality has been most successful with rice, which has led to five perennial rice lines being developed, one of which is in pre-release testing (Batello et al. 2013). None of these lines have been approved for commercial distribution yet. This success is most likely due to the fact that cultivated Asian rice, *Oryza sativa*, was domesticated from a perennial species, *O. rufipogon*. Some cultivated genotypes maintain a weak perennial phenotype with plants producing a ratoon crop under irrigated conditions (Sacks et al. 2003). None survive for multiple years though.

The greatest research strides have come from wide crosses between *O. sativa* and *O. longistaminata*, a wild perennial African species that propagates via rhizomes. Two dominant complementary QTL for rhizomes, *Rhz2* and *Rhz3*, have been mapped to chromosomes 3 and 4, respectively; an additional 15 minor effect QTL for rhizome formation have also been identified (Hu et al. 2003; Hu et al. 2011). *Rhz2* corresponds closely to rhizome QTL on linkage group C of *Sorghum propinquum*, a wild perennial relative of cultivated sorghum (Hu et al. 2003). *Rhz3* corresponds closely to a major rhizome QTL on linkage group D of *S. propinquum* as well as to a dominant rhizome QTL on linkage group 2a of perennial wildrye (Hu et al. 2003; Yun et al. 2014). Molecular markers were developed for the rhizome QTL to transfer the traits by multiple backcrosses to the cultivated parent. A perennial cultivar, PR23, was developed that maintained impressive yields over the course of three seasons under tropical lowland paddy conditions. It is currently undergoing pre-release testing in Yunnan Province in China (Batello et al. 2013).

It is important to note that there are many perennial *Oryza* species and not all propagate from rhizomes. For example, *O. rufipogon*, the perennial ancestor of cultivated rice, propagates via stolons, which are horizontal stems that can be above or below ground. However, it is generally thought that rhizomes are more stress tolerant than stolons, thus *O. longistaminata* was chosen as the perennial parent for breeding. Even so, Sacks et al. (2006) found that rhizomes and perenniality were not associated in *O. sativa*/*O. longistaminata* interspecific genotypes/cultivar full-sib family progeny, with 15% of plants surviving past one growing season without rhizomes. Sacks et al. speculated rhizomes may not be necessary for perenniality, but instead might help plants manage and endure abiotic stresses.

Sorghum

Rhizome, tiller, and regrowth QTL have been identified and mapped in an F_2 population from a cross between the cultivated annual *Sorghum bicolor* and *Sorghum propinquum*, a closely related wild perennial species (Paterson et al. 1995). Winter survival and regrowth was measured in the field in Texas; 92.2% of F_2 plants survived and 46.3% of BC_1 plants survived. Paterson et al. (1995) found that regrowth QTL were associated with rhizome and tiller QTL, suggesting all three may be needed for perenniality in *Sorghum*. Two of the rhizome QTL closely correspond to *Rhz2* and *Rhz3* found in rice, as mentioned previously (Hu et al. 2003).

Two overwintering QTL have been identified and mapped in an F_4 heterogeneous family population from *S. bicolor*/*S. propinquum*; one of these QTL overlaps with one of the rhizome QTL just described (Washburn et al. 2013). All of the plants that successfully overwintered had rhizomes; however, only 33% of the overwintering

variation could be explained by the presence of rhizomes. The authors suggested this may be due to either (1) rhizomes not being as important to the overwintering phenotype as previously thought, or (2) current rhizome phenotyping methods are not capable of fully describing rhizome traits. Both Paterson et al. (1995) and Washburn et al. (2013) measured rhizome traits using above-ground indicators; for example, rhizome number was estimated by counting the number of above-ground shoots produced from rhizomes. This method does not detect rhizomes that did not produce a shoot and does not measure rhizome depth.

Arabidopsis

A single gene, *PERPETUAL FLOWERING 1* (*PEP1*) is the primary regulator of perennial flowering in *Arabis alpina*, a close perennial relative of *Arabidopsis thaliana*. *PEP1* is an ortholog of the *A. thaliana* gene *FLOWERING LOCUS C* (*FLC*); it maintains polycarpy and initiates flowering after vernalization (Wang et al. 2009). However, vernalization does not seem to be required for perenniality in *A. alpina* since five independent mutations in *PEP1* have been found in five different accessions, all of which do not require vernalization to flower (Albani et al. 2012). Interestingly, most of the *PEP1* alleles have a tandem arrangement of a full-length and a partial copy of *PEP1*, which produce two differentially expressed full-length transcripts. Albani et al. (2012) speculate the complex transcriptional pattern of the locus may allow it to participate in more regulatory pathways, which gives rise to the complex phenotype of perenniality.

Present study

In this study, our objectives were (1) to create a linkage map for the F_2 population produced after crossing maize B73 and *Z. diploperennis*, (2) to identify and map QTL responsible for the perennial phenotype, and (3) to identify single nucleotide polymorphism (SNP) markers associated with perennial traits, including overwintering, observed in F_2 plants. We used genotyping-by-sequencing (GBS) technology to generate SNP markers for both parents and a subset of F_2 plants to create a linkage map and then used QTL mapping to identify significant relationships between perennial traits and particular SNPs.

CHAPTER 2

MATERIALS AND METHODS

Plant materials

Zea diploperennis (PI 462368**;** obtained from the North Central Regional Plant Introduction Station, USDA-ARS in Ames, Iowa) was used as the paternal parent and crossed with maize B73. Eight F_1 plants were grown and sib crossed via open pollination. On May 8, 2014, we planted 800 F_2 kernels, of which 482 survived to the end of the phenotyping period. We carried out phenotypic work on all 482 plants, and did genotypic work on a subset of 93 plants in addition to the parents. We also planted eight *Z. diploperennis* (also PI 462368) from seed in the field to record phenotypic characterizations that would be representative of the *Z. diploperennis* parent in its first growing season. However, all eight plants were shaded out by neighboring F_2 plants 4-6 weeks after planting. They did not show meaningful above-ground biomass until after cutting in September 2014. Two ramets of the *Z. diploperennis* parental genotype that were transplanted as fully mature plants in an adjacent field the previous year were used for phenotypic characterization as well. Unfortunately, information on *Z. diploperennis* trait development during the first year of growth could not be provided by these ramets, since they were in their second growing season. B73 plants were also planted in several places in the field to note the flowering time. B73 does not form tillers, produces 14-16 rows of kernels, and dies rapidly after seed set.

Field design

Kernels were planted in one of three blocks in a field (Figure 1) in Athens, Georgia. Each block contained *Z. diploperennis* plants at the northwestern corner—two plants in the western block, four in the middle block, two in the eastern block—with F_2 plants planted twelve inches apart in the remaining space. Plants were allowed to open pollinate over the course of the summer.

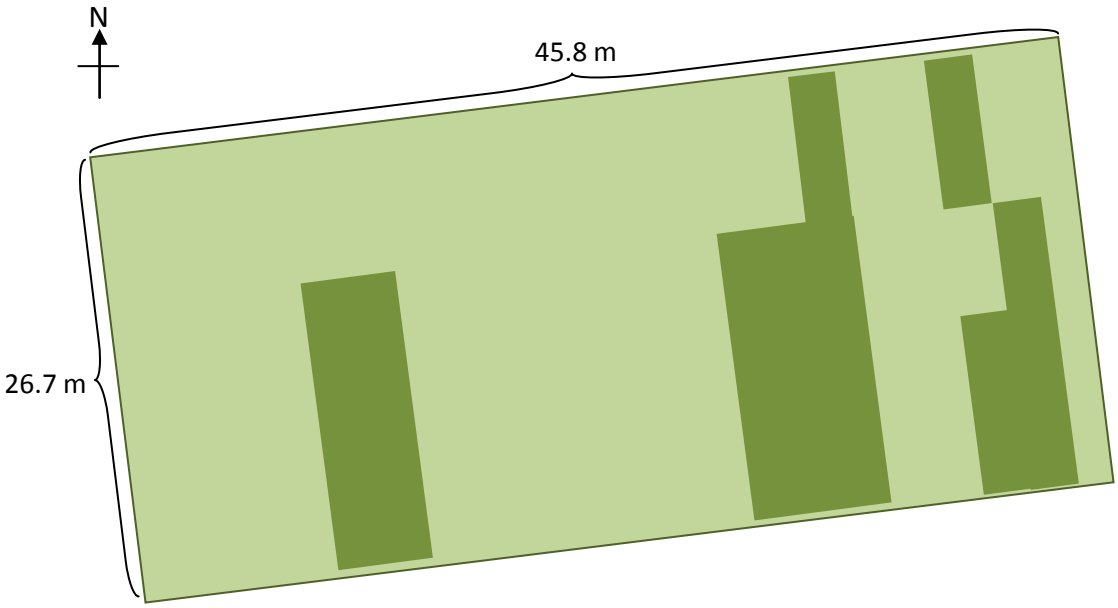

Figure 1. Field design for experiment. Kernels were planted outside in a field behind the UGA greenhouses in Athens, Georgia. The entire field is shown in light green. Plants were only grown in areas marked in dark green.

Soil analysis

Six soil samples were taken in January 2015. Samples were taken in evenly spaced intervals, with three samples taken in the northern half of the field and three samples taken in the southern half; sampling sites were not restricted to planting sites (dark green area in Figure 1). All samples were taken six inches deep in the soil and allowed to dry overnight before being sent to the Soil, Plant, and Water Lab, which is a

part of the Georgia Cooperative Extension and the Agricultural Experiment Station. A routine soil test was requested, which tested for soil pH, extractable phosphorous, potassium, calcium, magnesium, manganese, and zinc. The results were considered to be acceptable for growing maize in northeastern Georgia (Table 1), according to the Georgia Cooperative Extension (Kissel & Sonon 2008).

Table 1. Means and standard deviations (s.d.) for phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg), zinc (Zn), manganese (Mn), and pH levels for six soil samples from field. Soil samples processed by the Soil, Plant, and Water Laboratory of the UGA Ag & Environmental Services Labs (http://aesl.uga.edu/).

	P lbs/acre	K lbs/acre	Ca lbs/acre	Mg lbs/acre	Zn lbs/acre	Mn lbs/acre	pH
Mean	35	256	2,313	275	7	31	6.1
(s.d.)	(22.6)	(71.5)	(615.8)	(75.6)	(4.1)	(12.8)	(0.2)

Phenotypic analysis

Seven traits related to the difference in perennial versus annual habit between *Z. diploperennis* and maize B73 were analyzed in the parents and 482 F_2 plants. We recorded the time tassels, pollen, and silks were visible on plants. This information was recorded weekly throughout the summer until the end of August 2014. All plants were cut back with a machete to about eighteen inches in height over the course of September 2014, after which the total number of tillers was recorded for each plant. A tiller was defined as a lateral branch that connected to the plant via a below-ground node (de Leon & Coors 2002). Two weeks after cutting plants back, we recorded any signs of regrowth in the form of new, above-ground, green tissue. Four to eight weeks after cutting plants

back, we recorded any signs of plants "staying green," meaning any above-ground tissue was still green. Stay-green tissues were typically stalks and growth spots at nodes. Overwintering was scored in the spring of 2015.

Genotypic analysis

Genomic DNA was extracted from leaf tissue of 93 F_2 plants as well as from the leaf tissue of the maize B73 parent and from the root tissue of the *Z. diploperennis* (PI 462368) parent using a modified CTAB protocol (modified from a CIMMYT Applied Molecular Genetics Laboratory Protocol and based on Saghai-Maroof et al. 1984). All tissues were freeze dried before extraction, except for the maize B73 leaf tissue. All leftover freeze dried samples have been stored for future use. Only a subset of the 482 F_2 plants was genotyped due to available funds.

Two phenotypic groups were represented in the 93 F_2 plants analyzed (Table 2). The first was comprised of 44 plants that had completely flowered (produced tassels, pollen, and silks) by the end of August and showed regrowth or stay-green traits or both after cutting. This group is referred to as the "perennial hopefuls" and abbreviated PH. The second group is comprised of 49 plants that had completely flowered and showed no regrowth or stay-green traits after cutting. These plants also had 3-4 tillers per plant, which was the average number of tillers found in the PH group. This second group of plants is referred to as the "crispy tillers" and abbreviated CT.

Table 2. Labels and phenotypic groups of plants genotyped-by-sequence.

Plant Label	**Phenotypic Group**
Maize B73	Parent
Zea diploperennis (PI 462368)	Parent

CC14-222	Perennial Hopeful
CC14-223	Perennial Hopeful
CC14-224	Perennial Hopeful
CC14-235	Crispy Tillers
CC14-246	Crispy Tillers
CC14-251	Perennial Hopeful
CC14-252	Perennial Hopeful
CC14-264	Crispy Tillers
CC14-266	Perennial Hopeful
CC14-280	Crispy Tillers
CC14-284	Crispy Tillers
CC14-287	Crispy Tillers
CC14-297	Crispy Tillers
CC14-299	Crispy Tillers
CC14-302	Perennial Hopeful
CC14-311	Crispy Tillers
CC14-319	Perennial Hopeful
CC14-321	Perennial Hopeful
CC14-322	Crispy Tillers
CC14-326	Crispy Tillers
CC14-328	Perennial Hopeful
CC14-335	Crispy Tillers
CC14-336	Perennial Hopeful
CC14-348	Crispy Tillers
CC14-355	Crispy Tillers
CC14-356	Perennial Hopeful
CC14-357	Perennial Hopeful
CC14-359	Perennial Hopeful
CC14-366	Crispy Tillers
CC14-367	Crispy Tillers
CC14-381	Crispy Tillers
CC14-382	Crispy Tillers

CC14-388	Crispy Tillers
CC14-390	Crispy Tillers
CC14-394	Crispy Tillers
CC14-397	Perennial Hopeful
CC14-399	Crispy Tillers
CC14-400	Crispy Tillers
CC14-404	Crispy Tillers
CC14-407	Perennial Hopeful
CC14-412	Crispy Tillers
CC14-413	Crispy Tillers
CC14-418	Perennial Hopeful
CC14-419	Perennial Hopeful
CC14-420	Perennial Hopeful
CC14-427	Perennial Hopeful
CC14-432	Perennial Hopeful
CC14-439	Crispy Tillers
CC14-441	Perennial Hopeful
CC14-445	Crispy Tillers
CC14-446	Crispy Tillers
CC14-450	Perennial Hopeful
CC14-458	Perennial Hopeful
CC14-475	Perennial Hopeful
CC14-477	Crispy Tillers
CC14-481	Crispy Tillers
CC14-483	Crispy Tillers
CC14-485	Perennial Hopeful
CC14-488	Crispy Tillers
CC14-491	Crispy Tillers
CC14-492	Crispy Tillers
CC14-494	Perennial Hopeful
CC14-496	Crispy Tillers
CC14-497	Perennial Hopeful

CC14-505	Perennial Hopeful
CC14-506	Crispy Tillers
CC14-509	Crispy Tillers
CC14-513	Crispy Tillers
CC14-521	Perennial Hopeful
CC14-526	Crispy Tillers
CC14-528	Crispy Tillers
CC14-537	Perennial Hopeful
CC14-539	Crispy Tillers
CC14-540	Perennial Hopeful
CC14-541	Perennial Hopeful
CC14-544	Perennial Hopeful
CC14-545	Perennial Hopeful
CC14-549	Crispy Tillers
CC14-567	Perennial Hopeful
CC14-578	Crispy Tillers
CC14-580	Crispy Tillers
CC14-583	Perennial Hopeful
CC14-589	Crispy Tillers
CC14-593	Perennial Hopeful
CC14-600	Crispy Tillers
CC14-607	Perennial Hopeful
CC14-626	Crispy Tillers
CC14-645	Perennial Hopeful
CC14-653	Perennial Hopeful
CC14-654	Perennial Hopeful
CC14-655	Perennial Hopeful
CC14-656	Perennial Hopeful
CC14-658	Crispy Tillers

All DNA samples were sent to the Institute for Genomic Diversity (Cornell University, Ithaca, NY, USA) for 96-plex genotyping-by-sequencing (GBS) analysis using the *ApeKI* restriction enzyme and following the GBS protocol (Elshire et al. 2011).

Sequencing data analysis and SNP calling

Single nucleotide polymorphism (SNP) calls were made using the raw Illumina sequencing from the Institute for Genomic Diversity and custom scripts from the Devos Lab at the University of Georgia. Reads were aligned to the maize B73 reference genome (AGP version 3.25) before filters were used to discard SNPs that were not found in more than 85% of individuals, segregated distortedly due to technical reasons (deviated significantly from the expected Mendelian segregation ration of 1:2:1; determined using Chi-squared test, $p > 0.05$), had less than four reads per SNP per individual, or were not homozygous for one parent or the other. Originally, we had wanted eight reads per SNP per individual to be confident in heterozygous calls, but this stringency left too few SNPs to map with.

Genetic map construction

After filtering, the remaining SNPs were mapped using MapMaker 3 (Lander et al. 1987; Paterson et al. 1988; Lincoln et al. 1993; Lincoln 1992).The order of SNPs was based on their position in the B73 reference genome and recombination frequencies were determined using MapMaker 3. MapChart 2.2 (Voorrips 2002) was used to draw the genetic map. Distances between SNPs were determined in centimorgans (cM) using the Kosambi function. All markers were named with the prefix “M” (for “marker”).

Quantitative Trait Locus/Loci (QTL) mapping

Composite interval mapping (CIM) was done using the computer program QTL Cartographer for Windows (version 2.5_011, Wang et al. 2012) to map QTL controlling the perennial phenotype in the F_2 population. CIM was run with the default program setting for model 6 (5 background markers and a window size of 10 cM). LOD thresholds were determined from 500 permutations of the data at a walk speed of 2 centimorgans (cM) with the significance threshold set to 0.05 before mapping.

CHAPTER 3

RESULTS

Phenotypic characterization

Flowering

Tassel emergence, pollen shedding, and silks emergence were monitored starting after F_2 plants germinated. Flowering abruptly started nine weeks after planting, which is approximately the time maize B73 begins flowering in Athens, Georgia. F_2 plants flowered until the end of August (week 15), which is when flowering traits were no longer recorded because the plants had started to show signs of Southern corn rust blight infection, which was particularly severe in 2014. We calculated a histogram, the mean values with standard deviations, and 90% confidence intervals to describe the distribution and variation in the flowering phenotypes (Figure 2, Table 3). The mean range for flowering for the F_2 population was from 10.0 to 11.9 weeks, with missing data not included in statistical analysis. Instances of staggered flowering were observed, meaning a plant produced tassels on one stalk and then produce more a few weeks later on another stalk, if present, for example. This phenotype was not formally recorded though. Non-synchronous flowering between the male (tassels) and female (silks) of the same plant was also observed, though not recorded, for some F_2 plants. The maize B73 and *Z. diploperennis* parents do not have mistimed flowering but instead coordinate tassel emergence, pollen shedding, and silks emergence in a concerted manner. For the 93 F_2

plants that were genotyped, the distribution and mean values for tassel emergence, pollen shedding, and silk emergence dates were calculated (Figure 4). Mean values and standard deviations were comparable to those of the whole population.

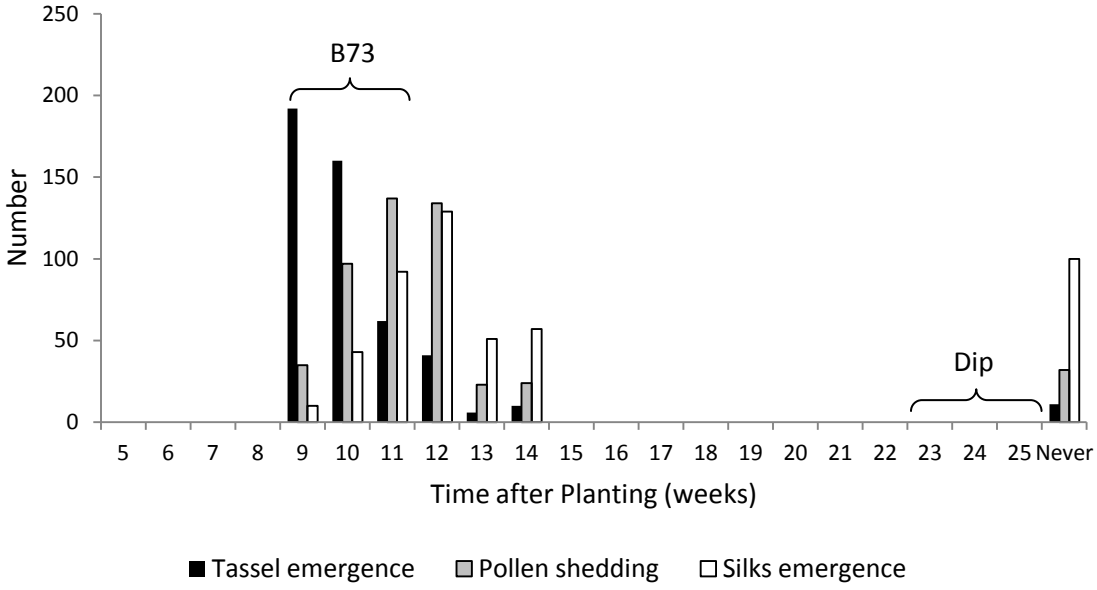

Figure 2. Distribution of flowering dates for 482 F_2 plants. Tassel emergence, pollen shedding, and silks emergence dates were recorded until the last week of August 2014 (week 15). The typical flowering cycle duration for the maize B73 parent is 10 weeks in Georgia. *Z. diploperennis* flowers in a day length dependent manner, and in Athens, Georgia it occurs in the second week of October. Parental flowering times (B73, Dip) are indicated with horizontal brackets. Only the first exhibition of these traits was recorded for each F_2 plant; no cyclical or multiple flowering events were recorded, although they did occur in some plants. Plants that did not exhibit these traits for the first time before the end of phenotyping were categorized as Never.

Table 3. Means, standard deviations (s.d.), and confidence intervals for flowering and tiller traits for 482 F_2 plants along with observed values for the eight *Z. diploperennis* individuals and maize B73 parent. Missing data was not included in the F_2 population statistical analysis.

Phenotype	Observed Values		Mean (s.d.)	90% Confidence Interval
	Z. diploperennis†	Maize B73	F_2 Population	
Tassel emergence	N/A	10	10.0 (1.2)	±0.1
Pollen shedding	N/A	10	11.2 (1.2)	±0.1
Silks emergence	N/A	10	11.9 (1.3)	±0.1
Tiller number	3-15	0	2.8 (2.1)	±0.2

†Flowering was only observed on the second-year *Z. diploperennis* ramets and the exact dates were not recorded.

Tiller number

The number of tillers was recorded immediately after plants were cut back in September 2015. Tillers were defined as lateral branches that connected to the plant at below-ground nodes (de Leon & Coors 2002). To describe the distribution and variation of the tiller number phenotype, we calculated a histogram, mean values with standard deviations, and 90% confidence intervals (Figure 3, Table 3). The mean number of tillers for the F_2 population was 2.8, which is between the parental values but skewed towards the maize B73 value of zero. Our F_2 mean is similar to those reported by Srinivasan and Brewbaker (1999). However, there was a significant difference in tiller number between plants in the eastern most planting group and plants in the western most planting group in the field ($p < 0.05$), with more tillers per plant observed in the eastern group. One reason for this effect may be that the tiller phenotype is environmentally influenced in grass species (Whipple et al. 2011). The two *Z. diploperennis* parental genotype ramets had an observed 16 to 18 tillers, but they were not in their first year of growth. The eight *Z.*

diploperennis individuals planted as seed in the spring of 2015 had a mean tiller number of 6.4 (s.d. ±3.8). While these plants are also a weak depiction of the *Z. diploperennis* first-year phenotype since all eight plants were shaded out until cutback, they are most likely a truer representation than the *Z. diploperennis* ramets. For the 93 F_2 plants that were genotyped, the distribution and mean values for tiller number were calculated (Figure 4). Mean values and standard deviations were comparable to those of the whole population.

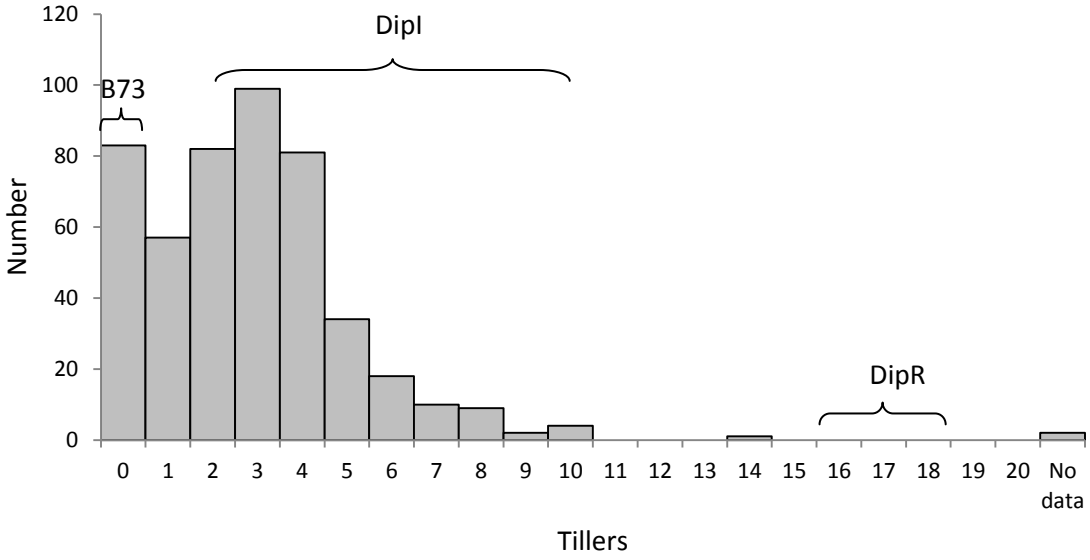

Figure 3. Distribution of tiller values for 482 F_2 plants. The values represent the number of tillers in addition to the main stalk of the plant. The typical values for the maize B73 parent (B73), *Z. diploperennis* individuals (DipI), and *Z. diploperennis* ramets (DipR) are indicated with horizontal brackets. Tillers were defined as lateral branches that connected to the plant at below-ground nodes (de Leon & Coors 2002).

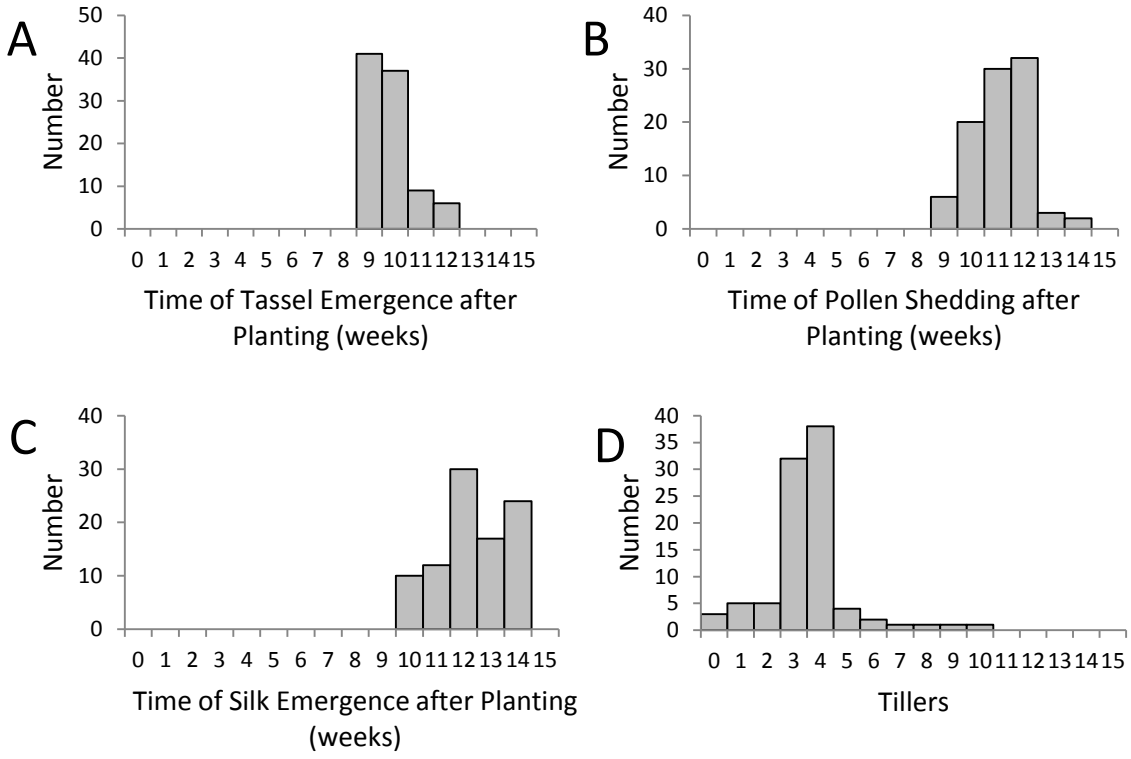

Figure 4. Distribution of tassel emergence, pollen shedding, silk emergence, and tiller number values for 93 F_2 plants that were genotyped (parents excluded). (A) The mean tassel emergence date among the genotyped F_2 plants was 9.8 weeks (s.d. ±0.9). (B) The mean pollen shedding date was 11.1 weeks (s.d. ±1.1). (C) The mean silk emergence date was 12.3 weeks (s.d. ±1.3). All three flowering traits were more representative of the maize B73 parent within the genotyped sample population. (D) The mean tiller number was 3.5 tillers (s.d. ±1.5), which is approximately equidistant between the maize B73 value (0 tillers) and the *Z. diploperennis* first-year growth mean value (individuals planted from seed; 6.4 tillers).

Regrowth, stay-green, and overwintering

Six F_2 plants, all eight *Z. diploperennis* individuals, and both *Z. diploperennis* ramets showed regrowth two weeks after being cut back in September 2014 (Table 4). Forty-three F_2 plants showed signs of stay-green tissue—typically stalks and above-ground nodes—four to eight weeks after cutback (Figure 5). One plant, CC14-223, exhibited both regrowth and stay-green traits. Regrowth and stay-green traits were only

considered legitimate if plants completed flowering and set seed before cutback. Annual species usually die shortly after setting seed; recording regrowth and stay-green traits in completely flowered and seeded plants helped ensure non-annual traits were studied.

None of the 482 F_2 plants showed regrowth in the spring of 2015 (Table 4). One of the eight *Z. diploperennis* individuals planted from seed (Dip #2) showed regrowth beginning in mid-March. Both *Z. diploperennis* ramets of the parental genotype showed regrowth at the same time. From October 2014 to mid-April 2015, there were 61 days with minimum air temperatures at or below freezing, the lowest of which was -12.1 °C on January 8, 2015. There was only one day the maximum temperature did not get above freezing (Figure 6). No soil temperature data was recorded.

Figure 5. Regrowth and stay-green phenotypes in F_2 plants after cutback. (A) An F_2 plant shows instances of regrowth (arrowheads) two weeks after cutback. (B) The F_2 plant on the left shows no signs of the stay-green trait, whereas the F_2 plant on the right has green stalks one month after cutting and is an example of the stay-green phenotype. There were two rounds of cutting and both occurred in September 2014.

Table 4. Fall regrowth and overwintering scores for parents, 482 F_2 plants, and *Z. diploperennis* controls.

Plant	Fall Regrowth	Spring Regrowth (Overwintering)
Maize B73	None	None
Z. diploperennis ramets	Yes	Yes
F_2 plants	Yes (7 of 482)	None
Z. diploperennis individuals	Yes	Yes (1 of 8)

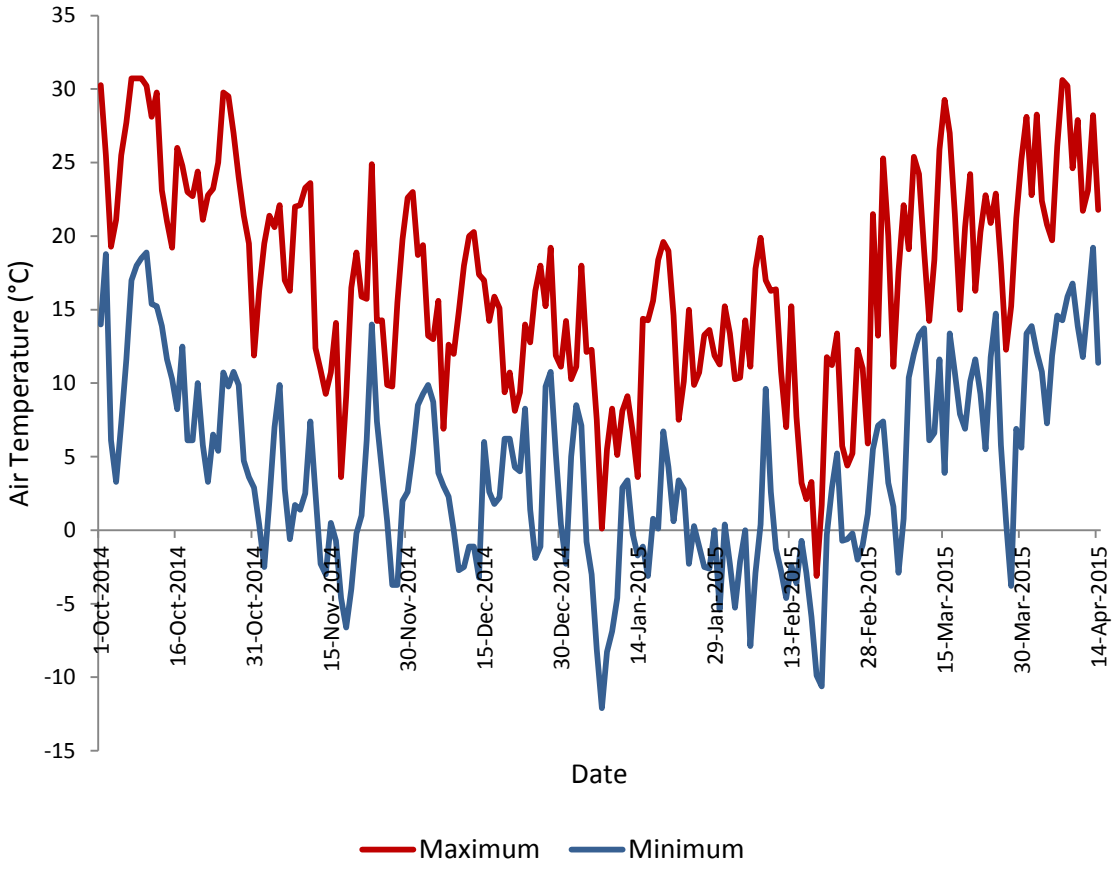

Figure 6. Maximum and minimum air temperatures from October 2014 to mid-April 2015. Weather data is for the location of Watkinsville, Georgia and is recorded by the Georgia Automated Environmental Monitoring Network (weather.uga.edu).

Genotypic characterization

Genotyping-by-sequencing (GBS) of the 93 F_2 plants and the two parents produced a total of $1.65x10^8$ 100-nt reads that passed all quality controls, with an average of 1,738,079 reads per barcode. One sample, CC14-246 of the Crispy Tillers (CT) phenotypic group, failed to generate reads. For the *Z. diploperennis* parent, a total of 3,345,166 100-nt reads were generated, which is approximately twice as many reads as any other sample. Since the *Z. diploperennis* parent is from a wild accession and therefore most likely heterozygous throughout much of the genome, we asked for extra read coverage to help ensure heterozygous SNPs could be called confidently.

A total of 7,668 SNPs were originally found after aligning sequence reads to the maize B73 genome and removing SNPs that either had fewer than four reads per SNP per individual or were not homozygous in one of the two parents. After further filtering to remove SNPs that were either missing in more than 15% of individuals or exhibiting high, non-biological segregation distortion, a total of 858 SNPs that were homozygous for contrasting alleles in either parent remained. These 858 SNPs were used to generate a genetic map (Figure 7, Figure 8).

The limited size of the genotyped samples (n = 93) and the occurrence of only two meioses (male and female) before production of the F_2 mapping population meant a restricted number of recombination events was able to be observed. The 858 SNPs were distributed across all ten chromosomes, ranging from 49 (chromosome 7) to 120 SNPs (chromosome 1) with an average of 85.8 SNPs per chromosome (Table 5). The mean distance between SNPs for each chromosome ranged from 1.1 to 2.6 centimorgans (cM) with an average of 1.7 cM for all chromosomes, resulting in a mean density of 0.6 SNPs

per cM for the genome. Chromosome 9 had the smallest distance between markers (1.1 cM) and the highest marker density (0.9 SNPs/cM), while chromosome 6 had the largest mean distance between markers (2.6 cM) and the lowest marker density (0.4 SNPs/cM). Segregation distortion due to biological factors was found, with 17.4% of SNPs (149 out of 858 SNPs) showing significant distortion ($p < 0.01$). A particularly large distortion region with all loci distorted towards the maize B73 alleles was found on chromosome 5 from position 10,224,187 to 216,043,427 bp (from marker M391 to M477), relative to the maize B73 reference genome (Table 6). Of the 100 SNP markers on chromosome 5, 76% showed very significant distortion ($p < 0.001$). The total length of the genetic map was 1,437.5 cM, which is 16.8% shorter than the genetic map for an F_2 mapping population with a similar marker density from two maize parents generated by Davis et al. (1999) (Table 5). The ten chromosomes had fairly good coverage and uniformity of marker distribution, with the largest distance between markers being 20.9 cM. The resulting map had no interval greater than 21 cM in length, and only 18 intervals were larger than 10 cM (Figure 7, Figure 8).

Table 5. Number of SNPs, map length, mean distance between SNPs, and density of SNPs per chromosome for genetic map of F_2 hybrids between maize B73 and *Z. diploperennis*. For map length comparison, genetic map length data from a maize x maize F_2 population is shown (Davis et al. 1999).

Chrom.	Number of SNPs	Mean distance between SNPs (cM)	Density (SNP/cM)	Length B73-Dip Coatney (cM)	Length Maize-Maize Davis et al. (cM)
1	120	1.6	0.6	192.7	245.2
2	102	1.5	0.7	155.6	200.2
3	85	2.0	0.5	166.6	164.8
4	69	2.0	0.5	138.8	169.7
5	100	1.5	0.7	150.3	174.8
6	49	2.6	0.4	126.0	168.6
7	90	1.7	0.6	152.9	147.5
8	74	1.7	0.6	123.6	167.6
9	100	1.1	0.9	111.4	150.4
10	69	1.7	0.6	119.6	138.6
Total	**858**	**1.7**	**0.6**	**1,437.5**	**1,727.4**

Table 6. Chi-squared significance values for segregation patterns of SNPs across all chromosomes. SNPs with no significance segregated in a Mendelian manner, whereas SNPs with greater statistical significance segregated in a distorted manner since they deviated from expected Mendelian segregation ratios (1:2:1). The observed segregation distortion was most likely due to biological factors (SNPs distorted due to technical factors were removed).

Chrom.	# SNPs with no significance	# SNPs with significance of $p < 0.05$	# SNPs with significance of $p < 0.01$	# SNPs with significance of $p < 0.001$	Total
1	118	2	0	0	120
2	80	7	8	7	102
3	76	8	1	0	85
4	40	22	6	1	69
5	14	4	6	76	100
6	48	1	0	0	49
7	48	29	12	1	90
8	36	24	11	3	74
9	58	27	10	5	100
10	50	17	2	0	69
Total	**568**	**141**	**56**	**93**	**858**
Percentage	**66.3%**	**16.4%**	**6.5%**	**10.8%**	**100.0%**

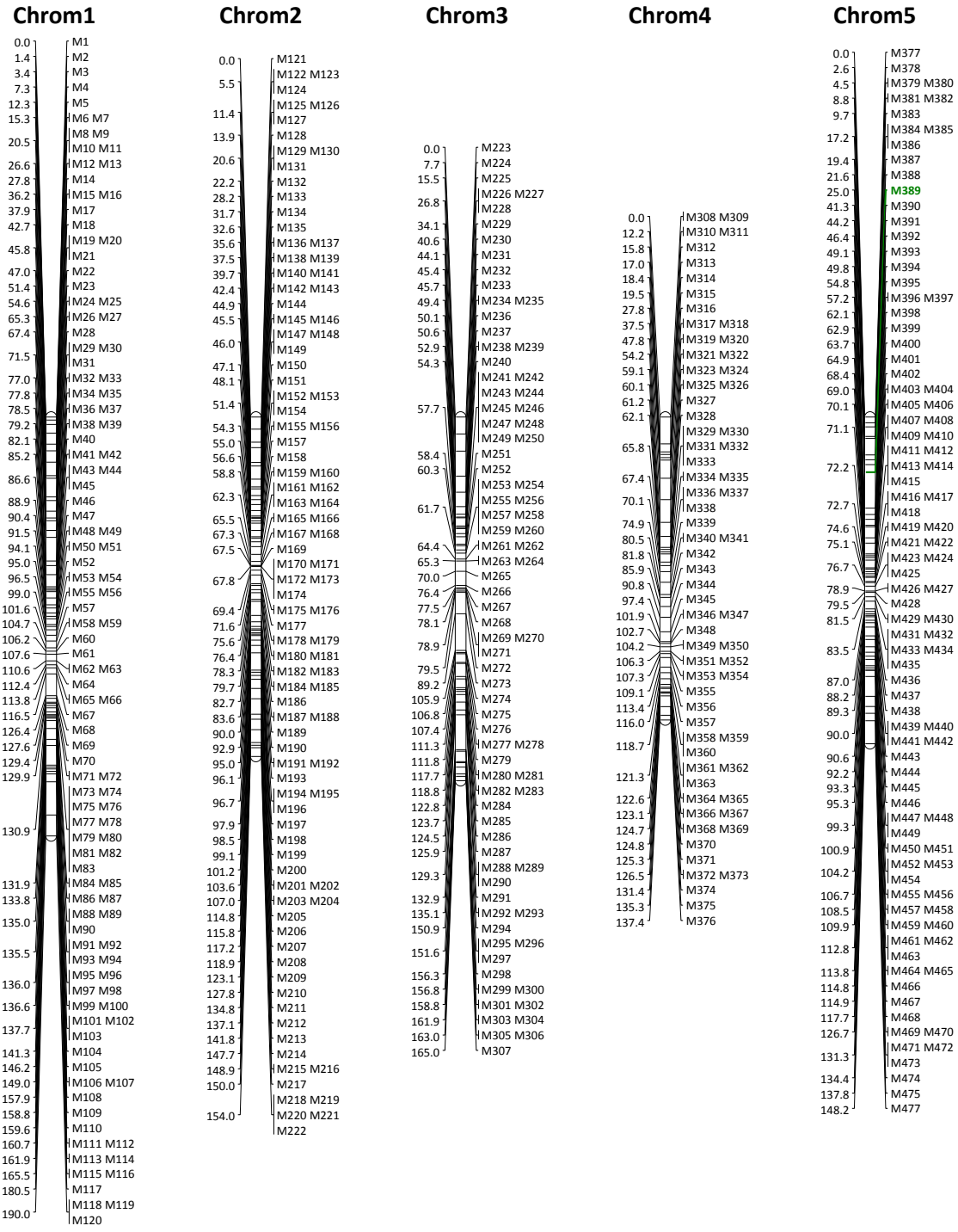

Figure 7. Genetic map for chromosomes 1-5 for F_2 mapping population. Numbers to the left of each chromosome are cumulative distances for each SNP marker (cM). Marker M389, which was associated with a stay-green QTL, on chromosome 5 is indicated in bold green text. Each marker is labeled MXXX on the right of each chromosome.

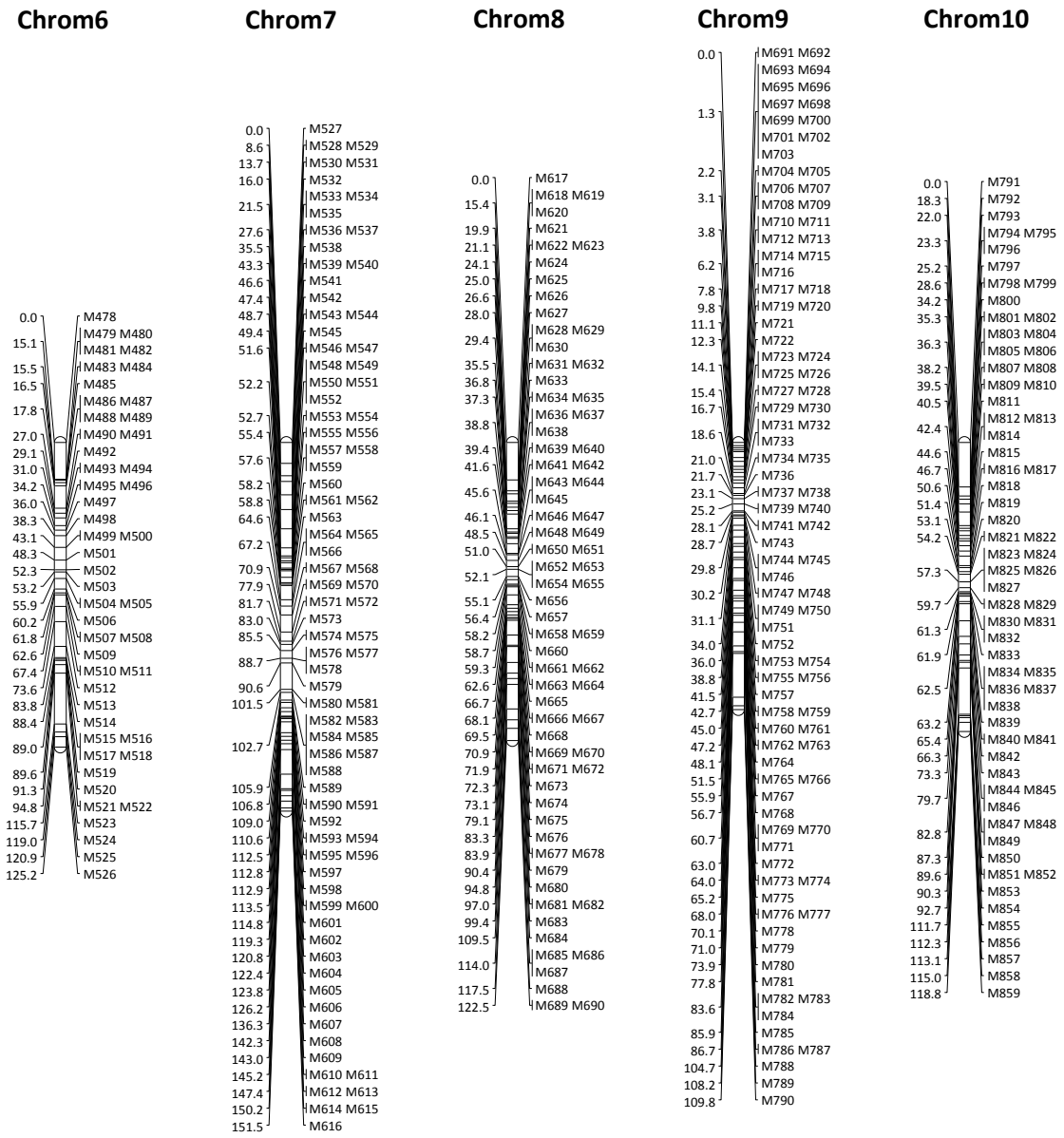

Figure 8. Genetic map for chromosomes 6-10 for F_2 mapping population. Numbers to the left of each chromosome are cumulative distances for each SNP marker (cM). Each marker is labeled MXXX on the right of each chromosome.

Quantitative Trait Locus/Loci (QTL) mapping

Composite interval mapping (CIM) found a single QTL for one of the seven perennial traits scored. With a LOD threshold of 4.1, a single QTL for the stay-green

phenotype was found on chromosome 5, which was associated with marker M389 (position 5:6364831on maize B73 reference genome) (Figure 9). M389 is located near the end of the short arm of chromosome 5 in maize B73 and in this particular F_2 mapping population was close to a recombination break point. While M389 was closely associated with the stay-green QTL peak, neighboring markers M388 and M390 also supported the peak with high, but not significant, LOD scores and were seen as shoulder peaks on the LOD graph (Figure 9). The physical distance between neighboring markers M388 and M390 on the reference genome is 5.29 Mbp, which covers approximately 443 protein coding genes (www.maizegdb.org). Interestingly, when only considering marker M389, the stay-green plants showed expected segregation ratios at this locus while the non-stay-green plants had distorted segregation towards the maize B73 allele ($p < 0.001$). The stay-green QTL explained 27.0% (r^2) of the total phenotypic variation. The additive and dominant effect values were -0.34 and 0.12, respectively, which suggested *Z. diploperennis* alleles contributed more positively to the effect than the corresponding maize B73 alleles (Table 7). No other significant QTLs were found for any of the other recorded traits.

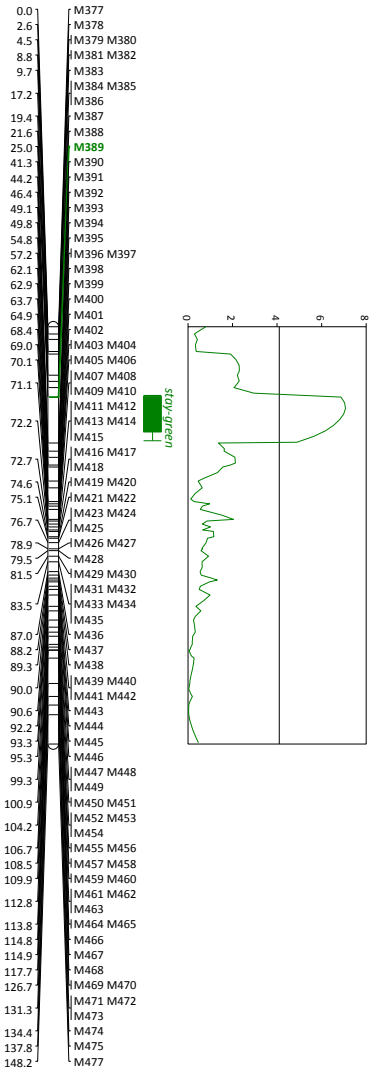

Figure 9. Genetic map of chromosome 5 plotted with QTL interval and QTL LOD graph for 93 F_2 plants. The QTL interval, represented vertically in green, is shown with 1 and 2 LOD intervals to the right of the map. The QTL LOD graph shows the LOD threshold of 4.1 for the stay-green phenotype. Marker M389, the most significantly associated marker with the QTL peak, is shown in bold green text.

Table 7. Chromosome location and direction of QTL for stay-green phenotype estimated by composite interval mapping (CIM) in 93 genotyped F_2 plants. *MML* nearest molecular marker loci, *a* additive effect, *d* dominance effect, *Dir* direction of the effect (whether the *M* maize or *D Z. diploperennis* allele contributed positively to the effect), r^2 proportion of phenotypic variation explained by QTL.

Chromosome	Position (cM)	MML	LOD	*a*	*d*	Dir	r^2
5	0.2501-0.4101	M389	6.9	-0.34	0.12	D	0.27

CHAPTER 4

DISCUSSION AND CONCLUSIONS

Failure to overwinter

Zero F_2 plants, one *Z. diploperennis* individual planted from seed, and both *Z. diploperennis* adult ramets transplanted to the field two years ago overwintered. It is unclear why the overwintering rate for the *Z. diploperennis* individuals was low while the adult ramets have successfully done so the past two years. It is known that rhizome development distinguishes perennial *Zea* species from their annual counterparts (Westerbergh & Doebley 2004). The data reported here raises questions about differences in rhizome development between first-year *Z. diploperennis* plants and older plants, assuming the shade effect the first-year plants experienced is not to blame. For example, did the shorter growing season in Athens, Georgia (relative to the native environment in central Mexico) stunt rhizome formation and thus hamper perenniality in the first-year plants? The adult plants were transplanted from the greenhouse as adults and did not experience a stunted growing season during the first year. Another factor to consider is if first-year rhizomes have different capabilities than older rhizomes. The potential for nutrient storage capacity may change with age. While differences in storage capacity might not impact *Z. diploperennis* in its native, tropical environment, these differences might influence the plant at northern latitudes. Rhizome depth has been shown to be important for successful overwintering for *Sorghum* in cold environments (Warwick et al.

1984; Warwick et al. 1986). Rhizome depth may increase with age, which would explain why the majority of the first-year plants failed to overwinter. These questions could easily be answered with a simple trait characterization study for first-year and adult *Z. diploperennis* plants in the field or greenhouse. Basic rhizome biology data would greatly improve experimental designs for future work similar to that reported here.

As for why none of the F_2 plants overwintered, those that produced rhizomes may have had the same fate as the majority of first-year *Z. diploperennis* individuals discussed earlier. However, additional factors concerning the influence of parental genomes should be considered. The maize B73 genome has experienced significant selection. There is also significant presence-absence variation between maize inbred lines (Springer et al. 2009), which might be further exaggerated between inbred lines and teosinte species. Together, the high degree of selection and presence-absence variation may cause maize B73 to lack key genetic factors or to be unable to contribute to important epistatic interactions to make the perennial phenotype possible in hybrid progeny. Maize B73 may be an unsuitable parent for a mapping population focused on uncovering the genetics of perenniality. Inbred maize lines in general may be unsuitable parents considering Srinivasan and Brewbaker (1999) used eleven different Hawaiian inbreds as mapping parents to no avail. An approach like the one used by Westerbergh and Doebley (2004), where the annual teosinte species *Z. mays* ssp. *parviglumis* was crossed with *Z. diploperennis*, may prove more fruitful (in their study, Westerbergh and Doebley (2004) did not score for overwintering). Indeed, researchers at South Dakota State University have found preliminary success recovering certain perennial traits in F_2 and F_3

populations after crossing *Z. diploperennis* with an annual Northern Flint variety (*Zea mays* cv. Rhee Flint) (Qiu et al. 2015).

Another possibility for the failure of all F_2 individuals to overwinter is the phenotypic threshold for overwintering was not met in any of the plants. Overwintering, while scored phenotypically as a binary trait (yes/no), is most likely a very quantitative trait genotypically and therefore considered a threshold trait. A certain genotypic threshold must be met for the overwintering phenotype to be present. It is possible the threshold was not met for any of the F_2 individuals; segregation distortion might have prevented important overwintering loci to be sufficiently assembled. The segregation distortion region on chromosome 5 completely distorted towards maize B73 alleles, for example.

Segregation distortion

Of the 858 SNP markers used to make the genetic map, 17.4% showed significant segregation distortion ($p < 0.01$). Segregation distortion has been found in a previous genetic map for a BC_1 population generated from a cross between maize B73 and *Z. diploperennis*. Wang et al. (2012) found 144 out of 320 (45%) simple sequence repeats (SSR) markers segregating distortedly. Specifically on chromosome 5, they found two segregation distortion regions and two gametophytic factors. This phenomenon has been reported before for intraspecies crosses within the *Zea* genus (Doebley & Stec 1993), as well as in other crop species.

Genetic map

The genetic map reported here has 858 SNPs across ten chromosomes spanning a total of 1,437.5 cM. This is 16.8% shorter than total length reported for a previous map built using an F_2 population derived from a cross between two maize inbred lines, Tx303 and CO159, and 1,736 markers of different marker types (Davis et al. 1999). The two maps are reasonably close to each other in length despite one is derived from an interspecies cross and the other from an intraspecies cross. Interspecies crosses typically have lower recombination rates and therefore generate shorter linkage maps.

One limitation to the approach reported here was mapping reads to the maize B73 reference genome. While the reference genome is a powerful resource, important information can be lost during wide hybridization crosses if no other resources are used. Only reads that matched closely enough with the reference genome were selected for analysis. Therefore, some structural variations in either the *Z. diploperennis* parent or F_2 progeny may have gone undetected based on our approach.

Perenniality genetics

A total of one QTL was detected for traits related to perenniality. None of the flowering traits had any significant QTL peaks, which is consistent with the literature (Buckler et al. 2009). Tiller number also did not generate any significant QTL peaks, but that was expected since nearly all of the genotyped plants were selected to have 3-4 tillers per plant. Plants that showed regrowth in the fall after cutback did not generate any significant QTL data; no plants successfully overwintered, so there was a dearth of information for this trait as well. However, plants that stayed-green a month after cutback

provided significant QTL data. A peak on chromosome 5 with a peak LOD score of 6.9 (significance threshold of LOD 4.1) was associated with the stay-green phenotype. This peak was linked to SNP marker M389, the thirteenth marker on chromosome 5, and was supported by neighboring markers M388 and M390. The neighboring markers were spaced from M389 by 3.4 cM and 16.3 cM, respectively. Additional markers were found in this region but were excluded from the final analysis due to missing data. Increased read coverage in the future would help resolve the size of this peak.

None of the maize research on perenniality scored for the stay-green trait. Withered stems, a trait potentially related to the stay-green phenotype, was studied by Westerbergh and Doebley (2004) in *Z. diploperennis* and *Z. parviglumis* F_2 progeny four months after planting; however, no phenotyping was done after seed set like in the study presented here. Some research has been done on the hereditary basis of stay-green traits in maize outside the context of perenniality. Fourteen QTL related to the stay-green phenotype were found in $F_{2:3}$ lines derived from a cross between maize inbred Mo17 (non-stay-green) and maize inbred Q319 (stay-green) using SSR markers and CIM (Zheng et al. 2009). Stay-green was scored as the percentage of green leaf area present and was measured at different time points after flowering. No QTL mapped to the same area as the stay-green QTL reported here. Before Zheng et al. (2009), Beavis et al. (1994) attempted to map QTL associated with stay-green (among other traits) as a proof-of-concept for using topcrossed and F_4 progeny to identify QTL. The way stay-green tissue was measured and scored was not specified. Three QTL were identified using interval mapping, but none were located on chromosome 5 and none were in agreement with those reported by Zheng et al. (2009).

In sorghum, one of maize's closest relatives, more research has been done to map stay-green QTL. Four stay-green QTL were first identified in 2000 on chromosomes 2, 3, and 5 in an F_7 RIL population (Xu et al. 2000). Phenotyping was during post-flowering drought stress with a visual scale of 1 to 5. These QTL were confirmed after more molecular markers were added to the analysis with one QTL, *Stg2* (chromosome 3), found to explain the most of the phenotypic variation (Subudhi et al. 2000). Additional stay-green QTL were found by Haussmann et al. (2002) after studying two different recombinant inbred populations and finding only three QTL were consistent between the two different genetic backgrounds over two experimental years. Stay-green tissue was measured in a similar manner to that of Zheng et al. (2009). These QTL were located on chromosomes 1, 7, and 10. None of these QTL studies were studying stay-green phenotypes in a perenniality context. Of the sorghum stay-green QTL mentioned here, only one found by Haussmann et al. (2002) mapped to chromosome 5 in maize. One of the flanking markers for the sorghum QTL was the RFLP marker umc166, which is located on the short arm of maize chromosome 5 and on the top half of sorghum chromosome 1. The stay-green QTL found in the data reported here and the umc166 marker are both located in the same syntenic block between maize and sorghum (Figure 10). The shared homology in this region lends support to the stay-green QTL data found here and invites future investigation.

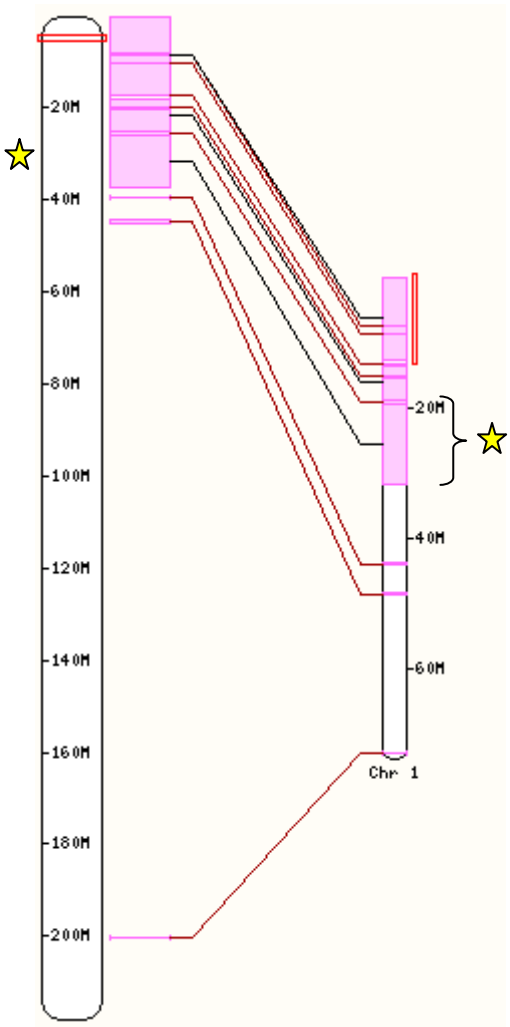

Figure 10. Synteny between maize B73 chromosome 5 (left) and *Sorghum bicolor* chromosome 1 (right) at location of stay-green QTL. Red boxes indicate the area between the flanking markers, M388 and M390, of the stay-green QTL on the maize chromosome and the homologous region on the sorghum chromosome; pink boxes highlight syntenic blocks; black and brown lines connect syntenic blocks with the same or reverse, respectively, orientation; yellow stars indicate location of umc166 marker. Image was generated on www.gramene.org with stars added.

There are significant limitations of the QTL analysis reported here. First, no environmental (multiple field locations) or genotypic (clones) replications were used. These types of replications help control for phenotypic variation that is not specific to genotype. To lend confidence to the data reported here, a follow-up experiment with

added replication—multiple field locations would most likely be easiest to achieve—should be done to see if the same results are produced. Second, the sample size of the experiment limited the findings. In addition to incorporating replication into future experimental designs, the sample size should be significantly increased. While 482 F_2 plants were phenotyped, only 93 F_2 plants were genotyped. The Beavis effect states that a sample size of 100 will overestimate the phenotypic variance of correctly identified QTL by 10-fold and will only have a 3% chance of detecting small QTL (Xu 2003). However, if 1,000 progeny are analyzed, phenotypic variance estimates are "fairly close" to their true values. Therefore the phenotypic variance reported here for the stay-green QTL is certainly too high. Lastly, the QTL mapping analysis used here is not appropriate for some of the trait data collected. A basic assumption of composite interval mapping (CIM), as well as for other standard mapping analyses, is that trait data is continuous. The flowering and tiller traits in this study are examples of continuous data. A range, or continuum, of values was possible to describe the phenotypic states. However, the regrowth, stay-green, and overwintering traits were scored as presence/absence traits. Only two values were used to describe the observed phenotypes and therefore did not generate continuous data. To perform CIM, the presence/absence scores were converted to 1/0 values and classified as continuous data in QTL Cartographer. While there most likely is a significant QTL on chromosome 5 as found with CIM because of its LOD score and presence of supporting neighboring markers, a better estimate of its size and effect could be obtained if a more appropriate analysis was used that took into account the binary nature of the presence/absence traits.

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