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The genetic basis of upland/lowland ecotype divergence in switchgrass (*Panicum virgatum)*

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ABSTRACT

The evolution of locally adapted ecotypes is a common phenomenon that generates diversity within plant species. However, we know surprisingly little about the genetic mechanisms underlying the locally adapted traits involved in ecotype formation. The genetic architecture underlying locally adapted traits dictates how an organism will respond to environmental selection pressures and has major implications for evolutionary ecology, conservation, and crop breeding. To understand the genetic architecture underlying the divergence of switchgrass (*Panicum virgatum*) ecotypes, we constructed a genetic mapping population through a four-way outbred cross between two northern upland and two southern lowland accessions. Trait segregation in this mapping population was largely consistent with multiple independent loci controlling the suite of traits that characterizes ecotype divergence. We assembled a joint linkage map using ddRADseq and mapped quantitative trait loci (QTL) for traits that are divergent between ecotypes, including flowering time, plant size, physiological processes, and disease resistance. Overall, we found that most QTL had small to intermediate effects. While we identified colocalizing QTLs for multiple traits, we did not find any large-effect QTLs that clearly controlled multiple traits through pleiotropy or tight physical linkage. These results indicate that ecologically important traits in switchgrass have a complex genetic basis and that similar loci may underlie divergence across the geographic range of the ecotypes.

INTRODUCTION

Biological species are able to occupy a vast array of environmental conditions through adaptations driven by natural selection. Local adaptation is characterized by native populations consistently having greater fitness at their home habitat in comparison to foreign transplants from different habitats (Kawecki and Ebert 2004; Leimu and Fischer 2008; Hereford 2009). The majority of plant species have been found to be locally adapted based on empirical studies of fitness responses in reciprocal transplant studies (Leimu and Fischer 2008; Hereford 2009). Yet, we still know very little about the role that the genetic architecture underlying locally adapted traits plays in shaping how organisms respond to the environment in terms of their performance and fitness (Savolainen *et al.* 2013; Tiffin and Ross-Ibarra 2014). Further, local adaptation can be constrained or confounded by gene flow, lack of genetic variation, genetic drift, and the genetic architecture of traits (Kawecki and Ebert 2004; Hereford 2009).

Over time, local adaptation to different habitats can contribute to the formation of distinct ecotypes. The divergence of ecotypes can eventually lead to speciation through the evolution of ecological reproductive isolation (Ramsey *et al.* 2003; Kay 2006; Lowry *et al.* 2008; Glennon *et al.* 2012). Ecotypes generally differ in suites (i.e. syndromes) of locally adapted traits from other populations (Turesson 1922a, 1922b; Clausen 1951; Lowry 2012; Ravinet *et al.* 2016). These important trait correlations that characterize ecotypic divergence can result from pleiotropy or tight genetic linkage (Conner 2002; Wright *et al.* 2013; Mills *et al.* 2014). Alternatively, the traits that underlie syndromes that characterize ecotypic divergence could be under independent genetic control and be correlated as the result of linkage disequilibrium caused by strong

75 correlational selection (Brodie *et al.* 1995). Understanding whether the suites of traits that
76 characterize ecotype divergence are caused by pleiotropy/linkage or independent loci requires
77 genetic crosses and quantitative trait analyses (Rogers and Bernatchez 2005; Hall *et al.* 2006;
78 Lowry, *et al.* 2015).
79
80 In plants, ecotype formation is frequently driven by divergence in soil water availability across
81 habitats with ecotypes adapted to more mesic habitats typically are larger in size and flower later
82 than ecotypes from drier habits (Clausen and Hiesey 1958; Porter 1966; Latta *et al.* 2007; Lowry
83 2012). *Panicum virgatum* (switchgrass) is an ideal system for studying the evolutionary genetic
84 basis of ecotype divergence. Switchgrass is a long-lived outcrossing C4 perennial grass native to
85 a large region of central and eastern North America and extending south into Central America. It
86 is a common species of the tallgrass prairie, utilized as a forage crop, and has been championed
87 as a bioenergy feedstock (Casler *et al.* 2011; Casler 2012; Parrish *et al.* 2012). Switchgrass
88 phenotypic diversity is characterized by two major ecotypes: "Upland" and "Lowland," which
89 are hypothesized to have descended from glacial refugia (McMillan 1959; Zhang *et al.* 2011b).
90 Molecular studies support the distinctness of the two ecotypes by estimating deep dates of
91 divergence between distinct upland and lowland haplotypes on the order of 0.7-1 million years
92 ago (Morris *et al.* 2011; Zhang *et al.* 2011b). Upland plants are typically found in drier soil
93 conditions than lowland plants which typically reside in riparian habitats. The two ecotypes are
94 easily distinguished by a suite of morphological differences, with lowland plants tending to have
95 fewer and larger tillers, erect growth, a compact crown, blue-green waxy leaves, and late
96 flowering time. Genetic variation in the cytoplasm is highly correlated with the divergence of the
97 ecotypes (Morris *et al.* 2011; Zhang *et al.* 2011b). Although the species is polyploid (4x-8x),

recent full-sib linkage studies indicate tetraploid switchgrass maintains preferential pairing and disomic inheritance (Okada *et al.* 2010; Lu *et al.* 2013). Tetraploids of each ecotype are largely reproductively compatible (McLaughlin and Kszos 2005) and putative hybrids are found in regions of co-occurrence (Zhang *et al.* 2011a; Lowry *et al.* 2014). Porter (1966) showed that upland and lowland ecotypes are locally adapted to their respective habitats through a reciprocal transplant experiment. The upland ecotype was found to be more drought tolerant and have higher nitrogen demand than the lowland ecotype, which is more tolerant to flooding. Numerous other transplant and field trials have demonstrated phenotypic and physiological differences between the two ecotypes (Wullschleger *et al.* 1996; Casler and Vogel 2004; Cassida *et al.* 2005a, 2005b; Barney *et al.* 2009; Yang *et al.* 2009; Cortese *et al.* 2010). For example, resistance to rust fungus infection has been found to be heritable and lowland populations are typically more resistant than upland populations (Eberhart and Newell 1959; Uppalapati *et al.* 2013).

In addition to ecotype divergence, adaptive phenotypic variation in switchgrass is driven by environmental variables that correlate with latitude. Classic research in switchgrass has demonstrated that phenological traits, including date of emergence, flowering time, and date of senescence are strongly correlated with latitude of origin in common garden experiments (McMillan 1959, 1965, 1967). Transplantation experiments and field trials have consistently demonstrated that moving genotypes north and south of their locations of origin results in a loss of fitness due to a suite of environmental factors (McMillan 1959, 1965, 1967; Porter 1966; Casler and Vogel 2004; Lowry *et al.* 2014).

Here, we developed a new outbred genetic mapping population to understand the genetic basis of adaptation to environmental factors that are divergent between northern upland and southern lowland ecotypes of switchgrass. The mapping population was formed through reciprocal crosses between four grandparents derived from different locations across the Great Plains of North America. Two of the grandparents are lowland accessions derived from the southern Great Plains, while the other two grandparents are upland accessions from the northern Great Plains (Figure 1). This balanced design, including upland/lowland cytoplasm, allows us to ask whether a shared set of loci are involved in adaptive divergence between southern lowland and northern upland populations or if different alleles and loci might be involved in reaching similar phenotypes in different local ecotype populations. We assessed this complexity through genetic mapping and characterizing allelic effects for quantitative trait loci (QTL) associated with ecotype divergence. Our results provide insight into the underlying genetic basis of adaptive ecotypic variation.

MATERIALS AND METHODS

Outbred Mapping Population: *P. virgatum* is an obligate outcrosser (Martínez-Reyna and Vogel 2002) and as such it is necessary to account for the fact that parental material will be genetically heterogeneous and thus generate many marker segregation types. We created a four-way phase-known (pseudo-testcross) population to evaluate the genetic architecture of upland/lowland traits in switchgrass. In this scheme, two sets of grandparents [*lowland*$_1$ x *upland*$_2$ & *upland*$_3$ x *lowland*$_4$] were crossed to create F_1 hybrids that were then reciprocally crossed to generate two large "outbred F_2" populations ($F_{12♀}$x$F_{34♂}$, $F_{12♂}$x$F_{34♀}$) of 200 progeny

each. We used four tetraploid grandparents in this design: Alamo ("AP13" genotype, the reference genome and southern Texas accession), West Bee Cave ("WBC3" genotype, a central Texas lowland ecotype), Summer ("VS16" genotype, a northern upland accession), and Dacotah ("DAC6" genotype, a northern upland accession). The Alamo and Summer grandparents functioned as pollen donors in crosses and therefore the two F_1 hybrids and their subsequent outbred families differ in that they contain either lowland WBC3 or upland DAC6 cytotypes (Figure 1). Given disomic inheritance, each outbred family can segregate up to four unique alleles donated by the grandparents. Phase can be resolved from the multi-generational information. Sampling multiple grandparental alleles increases the possibility of evaluating informative QTL and inspection of the allelic effects of QTL may provide insight into genetic heterogeneity in ecotype divergence. For example, do QTL alleles from WBC and AP13 exhibit similar or differing additive effects relative to VS16 and DAC6 QTL alleles? Finally, the cytoplasmic segregation also allows for investigation into cytoplasmic and cytoplasmic by nuclear QTL interactions.

Cultivation and Phenotyping: Seed from the reciprocal F_1 hybrid cross was germinated and initially planted in four-inch pots in a greenhouse at the University of Texas at Austin. At the two true leaf stage they were transferred to one-gallon pots using a potting mix of Promix (Premier Tech Horticulture, Riviére-du-Loup, Quèbec, Canada) and Turface (Profile Products, Buffalo Grove, IL, USA) in a ratio of 4:1. Plants were grown in a greenhouse under 16h days from January to June 2012. Each plant was scored for date to first flowering, from time of transfer to the one-gallon pot to anthesis. Plant height was also quantified, as the total length of tallest tiller, on the day of first flower. Leaf tissue for genomic DNA extraction was collected and frozen in

liquid N_2. After flowering, the pots were moved from the greenhouse to an outdoor field nursery. At the end of the growing season the plants were clonally divided, by splitting the crown into generally equal halves. These two replicates were repotted in the same soil mixture using one-gallon pots and maintained in the field nursery. One replicate of each of the mapping progeny genotypes and 20 replicates of each grandparent and F_1 hybrid were transplanted into the field at the experimental garden site at the Brackenridge Field Labs in Austin, TX in February 2014, while the other set of replicates was kept as backup tissue and maintained in the field nursery. The field planting was based on a randomized honeycomb design with 1.25 m interplant distances surrounded by a row of border plants to minimize edge effects. Weed barrier cloth (Sunbelt 3.2oz., Dewitt, Sikeston, MO, USA) was used to aid in establishment and minimize plot maintenance. The common garden field site was located in lowland switchgrass habitat associated with Colorado River floodplain (30.284138° N, -97.781632° W), where the soil type is Yazoo sandy loam.

The following traits were measured during the 2014 growing season for field grown plants: date to first flowering, chlorophyll content, specific leaf area, midday water potential, total number of tillers, single tiller mass, number of leaves on five total tillers, height, and pathogen susceptibility. Date to first flowering, hereafter, flowering date, is calculated as the number of days from emergence to first anthesis. We measured midday water potential (MD WP) using a Scholander pressure chamber (PMS Instrument Company, Albany, OR, USA) on a mature leaf. Water potential indicates the leaf water potential status, and when measured during the heat of the day, can indicate tolerance to water stress. Larger (more negative) water potential values indicate more water stress (Pérez-Harguindeguy *et al.* 2013). Relative chlorophyll content was

189 estimated using a chlorophyll SPAD meter (Konica-Minolta SPAD 502; Konica-Minolta,
190 Chiyoda, Tokyo, Japan). Three readings from a single mature leaf were taken and the mean value
191 recorded. Relative chlorophyll content, or leaf "greenness", was estimated by a corrected ratio of
192 transmitted light with wavelengths 940 and 650 nm (Markwell *et al.* 1995). Specific leaf area
193 (SLA) is the ratio of leaf area (mm^2) to dry leaf mass (g), the resulting value is a measure of leaf
194 density (Pérez-Harguindeguy *et al.* 2013). We recorded the area of three mature leaves using a
195 portable leaf area meter (LI-3000A, Li-Cor, Lincoln, NE, USA) and recorded the mass of the
196 same leaves after full desiccation. Larger values for the area to mass ratio indicate a thinner, less
197 dense leaf. Total plant height, single tiller mass, total number of tillers, and number of leaves on
198 five tillers were taken at the end of the growing season. Height was measured using a graded
199 measuring rod and tiller mass was calculated by averaging the dry weight of five mature tillers
200 stripped of leaves and inflorescences. We calculated phenotypic correlations among traits for the
201 grandparent ecotypes and recombinant mapping progeny using the Spearman rank method on
202 raw phenotype values. We used the Holm-Bonferroni correction for multiple testing (Holm
203 1979).
204
205 **Rust Susceptibility:** Infection by a fungal rust pathogen was observed in the mapping
206 population in the potted plants in 2013. Plants were treated with a fungicide (Daconil
207 GardenTech, Palatine, IL, USA) to aid in vigorous establishment of clonal replicates and all but
208 12 inches of above ground biomass was removed before dormancy. The plants were then planted
209 in the field for the following 2014 growing season. Physiological phenotypes (see above) were
210 measured on green, healthy leaves. At the end of the growing season, five mature tillers were
211 harvested and manually stripped of their leaves. Each leaf was then scored for presence of the

fungal pathogen using a qualitative four point rating, one- completely infected and brown in color, to four - no evidence of pathogen and green in color (Figure S1). The total number of leaves on all five tillers was recorded as well. Each plant was then given a percentage score for each leaf category. We then used a principal component analysis for the four percentile scores to find the major axis of variation for the trait. The first principal component explained 86.4% of the variation and was largely associated with the percentage score of completely infected leaves. We therefore used the scores for the first principal component (Rust PC1) as a proxy for a pathogen resistance phenotype.

Genotyping: Fresh leaf tissue was collected in the greenhouse, immediately frozen in liquid N_2, and stored at -80°C in the spring of 2013. The equivalent of 100 mg of wet tissue was then used for genomic DNA extraction with the MasterPure Plant Leaf DNA Purification Kit (Epicentre, Madison, WI, USA). The procedure was modified with an initial RNase A treatment and two subsequent ethanol washes. Final DNA was eluted in 30 µl TE buffer and quantified using the Broad Range spectrum kit of the Qubit 2.0 (Life Technologies, Carlsbad, CA, USA). Each extraction yielded approximately 30-200 ng/µl DNA.

Each plant was genotyped using a double-digest Restriction-site Associated DNA sequencing (ddRADseq) (Peterson *et al.* 2012) scheme. In brief, 300 ng of DNA was cut with *Eco*RI and *Sph*I enzymes. In-line barcodes were ligated onto the *Eco*RI cutsite, fragments were size selected on a Pippen Prep (Sage Science, Inc. Beverly, MA, USA) at a 300 bp +/- 30 bp range, then Illumina adaptors were ligated onto the *Sph*I cutsite. This produced nine multiplexed libraries with 48 individuals each. Libraries were sequenced on an Illumina 2000 HiSeq (San Diego, CA,

USA) at the Genome Sequencing and Analysis Facility in Austin, TX. Raw read quality was evaluated with FastQC (v. 0.10.1). Library demultiplexing was performed in Stacks (v. 1.06, Catchen *et al.* 2011). Sequencing resulted in ~1.6 million (mean = 1,626,329 ± 34,002.78 SE) raw reads per individual. Reads from each individual were then mapped to the *P. virgatum* v1.1 genome (DOE-JGI, http://phytozome.jgi.doe.gov) using BWA *mem* (BWA v. 0.7.9a, Li 2013; Li and Durbin 2010). Mapped reads were further processed in Stacks for genotype calling and allele designations. The two F_1 hybrids were designated as parents in the Stacks ref_map.pl pipeline and 140,561 markers were included in the catalog of markers for scoring genotypes. Each individual was then genotyped at ~ 38,000 (mean = 38,161.2 ± 670.0 SE) markers. The coverage for each marker was approximately 40x. Due to missing data the dataset was filtered for markers present in at least 50% of the individuals. We also filtered for segregation distortion by testing for significant deviation from Mendelian expectations and removing all loci where $P<0.00005$. This resulted in a high quality set of 1,348 markers available for subsequent linkage analysis.

Linkage Map Assembly and QTL Analysis: Outbred mapping populations are often analyzed in a pseudo-testcross approach, developing independent maternal and paternal linkage maps based on single dose markers that uniquely segregate in either parent (Haley *et al.* 1994). Here, we develop a joint linkage map based on the outbred full-sib family (CP) design using Joinmap (v. 4.1) and the multipoint maximum likelihood (ML) algorithm (van Ooijen 2011). This algorithm is unique in that it simultaneously estimates phase and recombination fraction and can utilize all possible segregation types in ordering markers and estimating marker interval distances. We first grouped markers into linkage groups using a conservative logarithmic odds-ratio (LOD) of 10.0. We then used a simple regression algorithm to order markers on each group

with the following settings: pairwise recombination frequency < 0.4, LOD > 3, with a Kosambi mapping function to calculate genetic distances. Markers that affected goodness of fit (mean chi-square >3) were removed. We then used ML to order and estimate mapping distances for the edited groups. This produced a finalized map with 1,281 markers.

We mapped QTL for phenotypic traits for the upland/lowland outbred mapping population using a stepwise multiple-QTL model fitting method as implemented in R/qtl (Broman *et al.* 2003; Manichaikul *et al.* 2009). A generalized linear model was used to correct for the effects of potting cohort for traits measured in the greenhouse. Field traits did not need a cohort correction and were quantile normalized to create a uniform set of normally distributed traits. All QTL scans were performed using a normal model and Haley-Knott regression based on dense 2 cM grid of pseudomarkers generated using the calc.genoprob function. We calculated LOD penalties for main effects and interactions for each trait through 1000 permutations of the scantwo function at an α of 0.05. We conducted a forward/backward stepwise search for models with a maximum of 10 QTLs that optimized the penalized LOD score criterion. We calculated the 1.5 LOD drop interval of QTLs in the best-fit models and the percent variance explained for each QTL based on the final best-fit models using the fitqtl function. We designated cytoplasm (cross direction) as an additive and interactive covariate for the scantwo penalty calculation. We also designated cytoplasm as an additive covariate for the stepwise model fitting and performed a post-hoc nuclear by cytoplasm interaction test. Computation was performed on the Lonestar cluster at the Texas Advanced Computing Center at UT Austin (www.tacc.utexas.edu) using custom scripts (github.com/ermilano/4way).

Data Availability: Raw sequence reads are available at the NCBI SRA, accession number: SAMN05609456 <http://www.ncbi.nlm.nih.gov/biosample/5609456>. File S1 contains barcode information. File S2 is an R/qtl file that contains the joint genetic map and genotype and phenotype data for each individual in the QTL analysis. File S3 contains metadata for File S2.

RESULTS

Trait Variation, Divergence, and Correlations: Upland and lowland ecotypes of switchgrass are characterized by divergence in a variety of traits including differences in flowering time, growth architecture, physiological characteristics, and disease susceptibility (McMillan 1964, 1965, 1967; Casler *et al.* 2011; Uppalapati *et al.* 2013). Most traits we measured showed the expected trait differentiation between the upland (U) and lowland (L) ecotypes based on the mean values of each of the grandparents including: height (L>U), tiller mass (L>U), specific leaf area (U>L), rust resistance (L>U), flowering time (L>U) and leaf number (L>U) (Figure S2). SPAD and midday water potential did not differ between upland and lowland ecotypes, and tiller count was in the opposite direction of the pattern anticipated – lowland genotypes developed more tillers than upland genotypes during this establishment year. The phenotypic distribution of the traits in the F_1 individuals and the F_2 mapping progeny was generally unimodal and exhibited limited transgressive segregation (Figure S2). For example, flowering time in the F_2 progeny ranged from five to 78 days, surpassing the maximum grandparental ecotype value of 64 days from lowland AP13. We did not observe a significant effect of cytoplasm on any of the measured phenotypes (ANOVA $P > 0.05$ for all trait comparisons across cytoplasmic backgrounds, data not shown).

Ecotypes are comprised of suites of divergent phenotypes associated with adaptation to contrasting habitats. In the grandparents, we expected traits to be correlated with each other as a result of ecotypic divergence. For example, there is a positive relationship between flowering date and height due to short stature and early flowering date of the upland ecotype compared to the tall stature and later flowering date of the lowland ecotype. All correlations were positive except with specific leaf area, where the correlations were strongly negative. This is expected as the smaller, thinner upland leaves have a larger leaf area to leaf mass ratio. Genetic recombination can reduce the number of pairwise trait covariances if the traits are largely polygenic and not controlled by a few pleiotropic loci of major effect (Conner 2002). We calculated a correlation coefficient for all pairwise trait combinations in the progeny and found 14 significant correlations out of 36 total (Table 1). We found that some traits remained highly correlated after recombination, for example flowering date and height measured in the field (Figure 2a). Whereas other trait pairs do not have a significant relationship, for example flower date and specific leaf area (Figure 2b).

Linkage Map: We successfully ordered 1,281 ddRADseq markers on 18 distinct linkage groups, corresponding to the nine chromosomes of the tetraploid switchgrass N and K sub-genomes (Figure 3). The total map distance is 2,288.7 cM, with an average inter-marker map distance of 1.8 cM (+/- 0.55 SE). This map length is comparable to the "Kanlow" by "Alamo" pseudo-testcross map at 2,200.4 cM (Lowry, Taylor, *et al.* 2015), but larger than separate male and female F_1 maps ("Alamo" male (1,515 cM) and "Kanlow" female (1,935 cM), (Okada *et al.* 2010); "Alamo" female (1,733 cM) and "Summer" male (1,508 cM) maps (Serba *et al.* 2013)).

One third of the ddRADseq markers mapped to unanchored contigs in the *P. virgatum* reference genome, and we were able to place these markers onto linkage groups. There are several marker segregation types that result from an outbred cross depending on the heterozygosity in each of four grandparents (Wu *et al.* 2010). For example, four unique grandparental alleles <abxcd> result in a fully informative marker for both linkage and QTL mapping. However, loci with fewer than four unique alleles result in partially informative markers (e.g., <efxeg>). We found 798 partially informative bi-allelic markers, 450 partially informative tri-allelic markers and 33 fully informative markers. The fully informative markers uniquely identify the contributed grandparental chromosomes and were used to phase each linkage group for analysis of allelic effects.

QTL: Overall, we identified 33 QTL and three epistatic interactions across 11 traits using stepwise model selection (Figure 3). The largest additive effect QTL was located on linkage group (LG) 5N at 116.12 cM with 19.3 percent variance explained (PVE) for flowering time in the greenhouse. The largest additive effect QTL in the field were for specific leaf area on LG 9N at 36.63 cM (13.46 PVE) and plant height on LG 2K at 132.27 cM (13.34 PVE) (Table 2). The only trait lacking significant QTL was midday water potential. Notably, we found a total of seven QTL on LG 9K. Over two thirds of the QTL peaks were found on one third of the linkage groups, with 72.7% of the QTL localized to the tetraploid homeologue pairs 2, 5 and 9. We did not detect any significant ($p > 0.05$) cytoplasmic or nuclear by cytoplasmic interactions.

We found six QTL and one epistatic interaction for specific leaf area, the largest number of QTL per trait in our study. Together, these QTL totaled 57.23 PVE for that trait. We also found five

QTL for both measurements of height, in the greenhouse and in the field. Interestingly, there was only one region, on LG 9N from 120 to 130 cM, where QTL for both height measurements colocalized. We detected three and four QTL for flowering time in the greenhouse and in the field respectively. However, while each had a QTL on LG 2K, the confidence intervals did not overlap. We discovered three pairwise epistatic interactions that explained a moderate amount of variance relative to the additive effects. These interactions were found for height (8.12 PVE), specific leaf area (9.43 PVE) and greenhouse flowering time (11.22 PVE). We found significant rank changes in the allelic effects depending on genetic background within each case but there was no consistent pattern across the three separate interactions. Three traits, leaf number, SPAD, and rust resistance, yielded a single QTL, but each QTL was less than 10 PVE, suggesting there may be many undetected loci controlling these traits. However, we acknowledge the limitation of using single replicates of each genotype. Power to detect small effect loci can be increased with more replication, especially for low heritability traits.

Allelic Effects: The use of an outbred mapping population affords a more detailed evaluation of QTL allelic effects than traditional inbred line crosses given the contribution of potentially four grandparental QTL alleles in the F_2 progeny. After phasing, the allelic affects at QTL can be evaluated based on the four possible recombinant progeny including upland homozygotes (VS16/DAC6), lowland homozygotes (AP13/WBC3), and the two upland/lowland hybrid genotypes (VS16/AP13; DAC6/WBC3). Inspection of the additive effects can therefore allow some interpretation of the distribution of functional alleles within and between ecotypes and the genetic mechanism associated with each trait. For example, the functional alleles can be consistent between ecotypes where unique upland and lowland genotypes contribute the same

functional alleles as determined by the magnitude and direction of their additive effects (Figure 4a). Or functional alleles can be polymorphic within ecotypes, where individual genotypes contribute unique functional alleles to progeny irrespective of ecotype – a primary indication of this pattern would be the observation of strongly differing phenotypes in the two unique upland/lowland heterozygotes at a particular QTL (Figure 4b). We can also evaluate whether the direction of the allelic effects is the pattern expected given the overall phenotypic divergence between upland/lowland ecotypes. We found 10 of the 33 QTL had additive effects in the opposite direction of that expected for divergence between upland and lowland ecotypes (Table 2). For example, lowland ecotypes consistently flower later than uplands, but the lowland alleles for flowering date on LG 2K resulted in an earlier flowering time than upland alleles (Figure 4c). All five QTL on LG 2K, three out of four QTL on LG 5K, and one each on LGs 9N and 6N exhibited allelic effects in the opposite of expected upland/lowland ecotypic divergence.

DISCUSSION

To understand the genetic architecture of divergence between upland and lowland ecotypes of switchgrass, we assembled a genetic linkage map and conducted QTL mapping for a reciprocal four-way cross. The results of QTL mapping allowed us to then characterize functional allelic effects for traits associated with ecotype divergence. Segregation of phenotypic variation within the mapping population suggests that the suite of traits characterizing ecotypic divergence is the result of complex genetic architecture that involves limited evidence for any large effect or pleiotropic loci. QTL mapping also supported this conclusion, with loci controlling trait divergence distributed throughout the genome. We identified multiple QTLs with additive effects

that are consistent with patterns of ecotypic divergence. This result suggests that the some of same loci may be involved in upland/lowland ecotype divergence across the geographic range of switchgrass. Overall, our results provide a better understanding of the genetic architecture underlying ecotype divergence and set the stage for improvement of regionally adapted cultivars of switchgrass.

The Evolution of Ecotypes: Ecotype divergence is typically characterized by a suite of trait differences that are correlated with environmental conditions that compose habitats (Clausen and Hiesey 1958; Lowry 2012). Ecologists often focus on documenting similar suites of trait that are correlated across genotypes and species, which they refer to as trait syndromes (Tjoelker *et al.* 2005; Possen *et al.* 2015). However, without genetic approaches it is not possible to determine whether trait syndromes are driven by pleiotropy/linkage or independent loci with allelic variation structured across ecotypes. Allelic variation can become structured between ecotypes through a buildup of linkage disequilibrium (LD) across physically unlinked loci as a result of strong divergent and correlational selection. In a scenario where there is little to no gene flow between ecotypes, and selection is acting on suites of beneficial traits, we expect LD to build between loci associated with all traits that contribute to local adaptation. We also expect to see a significant reduction in LD and subsequent correlational structure following gene flow between ecotypes in nature or through the controlled crosses in our mapping study. In our mapping population, we found that 11 of the trait correlations that were significant in the grandparents were no longer significant in the F_2 generation, suggesting that those trait correlations were the result of LD due to population structure between the ecotypes.

While much of the trait syndrome that characterize the upland and lowland ecotype divergence was due to independent loci, we also found evidence of tight physical linkage and/or pleiotropy. Tight physical linkage and pleiotropy can both facilitate and constrain adaptation, depending on the direction of additive effects on different traits. If allelic effects across traits are in the direction consistent with local adaptation, then tight linkage and pleiotropy can facilitate ecotype formation (Mills *et al.* 2014; Schwander *et al.* 2014). However, if allelic effects on traits are in the opposite direction of local adaptation, then the evolution of these traits will be constrained due to genetic trade-offs (Kawecki and Ebert 2004; Savolainen *et al.* 2013; Tiffin and Ross-Ibarra 2014). In our study, we found that tiller mass and SLA as well as height and SLA were significantly correlated in both the grandparents and the mapping progeny. The raw correlation value was negative but this is the expected direction for ecotype divergence. Tiller mass and SLA shared overlapping QTL confidence intervals on LG 9K and both had effects in line with ecotypic divergence. Thus, physical linkage or pleiotropy may facilitate adaptive evolution of these traits. In contrast, height and SLA on LG 5K differed in direction of allelic effects. This genetic architecture could constrain response to selection along the axis of ecotype divergence.

Functional genetic variation is also often correlated with latitude, which reflects adaptation to environmental gradients that track latitude (Langlet 1971; Adrion *et al.* 2015). In switchgrass, much of the functional genetic variation has been shown to be strongly correlated with latitude (Casler and Vogel 2004; Casler *et al.* 2007; Lowry *et al.* 2014). The design of our study confounds the effects of ecotype and latitude because we only included northern upland and southern lowland individuals as grandparents in our crosses. While the lowland ecotype does not occur in northern regions, the two ecotypes overlap each other over a great portion of their range,

442 with upland populations occurring at least as far south as Texas (Lowry *et al.* 2014). To fully
443 partition latitudinal and ecotype effects, mapping populations would need to be made between
444 southern lowland and southern upland plants.
445
446 **QTL Co-Localization:** Our QTL findings are consistent with other *P. virgatum* studies and we
447 provide possible evidence for genomic regions across the *Panicum* complex with consistent and
448 conserved effects on traits. We found several regions in the genome where QTL confidence
449 intervals overlapped across traits, mainly on tetraploid homeologous pairs 2, 5, and 9. LG 2K is
450 particularly interesting because all of the QTL effects were in the opposite direction than
451 expected based on patterns of ecotypic divergence. This may be due to several factors that we
452 address below (see: *Allelic Effects)*. We found seven QTL that localized to LG 9K. This is
453 consistent with previous mapping efforts of similar traits by Serba et al. (2014) and Lowry et al.
454 (2015). Both studies found major biomass QTL on LG 9K.
455
456 Another interesting comparison is to QTL recently identified in the diploid relative of
457 switchgrass, *Panicum hallii*. The reference genome for switchgrass is anchored on the *P. hallii*
458 genome and switchgrass and *P. hallii* are estimated to be 5.3 million years diverged (Zhang *et al.*
459 2011b). *P. hallii* has two genomic hotspots of ecotype divergence, where QTL for many traits
460 associated with ecotype divergence colocalize on LG 3 and 5 (Lowry *et al.* 2015). LG 5 is
461 particularly interesting because QTL for tiller number, flowering time and height colocalized to
462 the lower arm of LG 5 in *P. hallii* and LG 5K in switchgrass. Additionally, the lower arm for
463 switchgrass LG 5N contained greenhouse-specific QTL for flowering time and height.
464

The earliest polyploidization in switchgrass was estimated to be roughly coincident with ecotype divergence ~ 1.2 million years ago (Zhang *et al.* 2011a). We found that specific leaf area had two QTL on the lower arms of LG 5K and 5N and the upper arms of LG 9K and 9N suggesting loci may have retained function across subgenomes rather than diverged or degenerated as a result of genome duplication.

Allelic Effects: Since upland and lowland ecotypes are estimated to have diverged 0.7-1 million years ago (Morris *et al.* 2011; Zhang *et al.* 2011b) during the Pleistocene we might expect the majority of functional alleles to be fixed between ecotypes because of putatively strong and consistent divergent selection pressures. However, if there were very low levels of gene flow across the geographic range of switchgrass populations we might expect functional alleles to be unique and varied among populations of each ecotype. This is consistent with a model of ecotype divergence stemming from ancient glacial refugia followed by adaptive radiations and genetic bottlenecks with each glacial cycle (Zhang *et al.* 2011b) and occasional gene flow across ecotype boundaries. In this model, ecotypes were originally formed from strongly selected and possibly small populations. However, there have been several glacial episodes since the estimated time of ecotype divergence, and with that, additional population bottlenecks and subsequent bouts of range shifts and changes in environmental selection pressures. Overall, we found that half of the QTL exhibited a pattern suggestive of fixed differences between ecotypes, supporting a model of considerable allelic heterogeneity both between and within upland and lowland ecotypes. Additional crosses and genetic mapping populations sampling a larger geographical distribution of both uplands and lowlands will be valuable for further insight into the evolution of switchgrass ecotypes.

Another important aspect of characterizing QTL is the direction of allelic effects. Even though QTL studies are known to both over and under estimate effect size of QTL (Beavis 1998) there is very little bias in detecting direction of effect. We found that 30% of our QTL had allelic effects in the opposite direction than expected for upland and lowland ecotype divergence. Here we offer some possible explanations. In addition to the directional selection imposed by heterogeneous habitats, populations may experience stabilizing selection, or maintain polymorphisms in populations in the overlapping ranges that experience higher levels of gene flow. In the case of flowering time and height, two traits that are strongly divergent between the ecotypes, we found half of the allelic effects were in the opposite direction of expected ecotype divergence. One possibility in this case is that the trait is composed primarily of many very small effect loci with allelic effects in the expected direction that are beyond the level of detection of this study. We found one particular region on the lower arm of LG 2K where all effects were in the opposite direction of ecotypic expectation. This may be the result of a chance fixation of a maladaptive chromosome block due to genetic drift in a population bottleneck (Orr 1998) or from a recent selective sweep for a trait we did not measure.

Pathogen Resistance: Rust infections of switchgrass pose major challenges to biofuel production as they can reduce ethanol yields up to 55% (Sykes *et al.* 2015). It is well known that the lowland switchgrass ecotype is more resistant to rust than the upland ecotype (Hopkins *et al.* 1995; Uppalapati *et al.* 2013). Fungal pathogens are generally moisture and temperature sensitive and often require high relative humidity for infection and sporulation (Harvell *et al.* 2002). Thus, a higher pathogen load could have driven greater resistance in southern lowland populations.

511 Whatever the cause, little is known about the genetic architecture of this resistance. The natural
512 infection that occurred during our experiment allowed us to screen for resistance QTL. We
513 detected one resistance allele from the lowland WBC3 genotype using our coarse scale
514 phenotype method. This lays the groundwork for future pathogen resistance mapping efforts in
515 switchgrass that can be used to develop a panel of resistance alleles. Marker assisted breeding
516 programs can quickly and efficiently develop cultivars for different climactic regions for traits
517 not amenable to transgenic manipulation and genetic engineering. Locating causal genes within
518 QTL is costly, time intensive and not useful if the phenotypic effect of each QTL is small. Yet it
519 would be possible to introduce resistance alleles into the upland genetic background through
520 several rounds of targeted marker assisted breeding (Vogel and Jung 2001).
521
522 **Conclusions:** Overall, our study has established a better understanding of the genetic
523 architecture of ecotype divergence in switchgrass. In addition to a conceptual framework for
524 genetics of locally adapted ecotypes, we provide a starting point for marker-assisted selection of
525 desired traits in a lignocellulosic bioenergy feedstock. Understanding and exploiting locally
526 adapted traits in different genotypes will allow us to efficiently grow switchgrass in many
527 different geographical regions economically and with minimal input and ecological impact.
528 Since there appears to be only limited pleiotropy underlying the divergence of upland and
529 lowland ecotypes, it should be possible to improve many traits through breeding without
530 incurring major phenotypic costs in other traits.
531
532 Going forward, we plan to utilize the four-way mapping population to better understand genetic
533 architecture of local adaptation across different latitudes. Clonal replicates of this mapping

population are currently being planted in common gardens across the latitudinal gradient of the North American Great Plains to address clinal variation and genotype x environment interactions in *P. virgatum*. These complex interactions, in addition to ecotype divergence, are important considerations in habitat restoration, plant breeding, and accuracy of agronomic modeling.

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AUTHOR CONTRIBUTIONS

T.E.J and D.B.L designed and funded research, D.B.L performed crosses, E.R.M performed research and analysis, and all authors wrote the paper.

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733

Tables

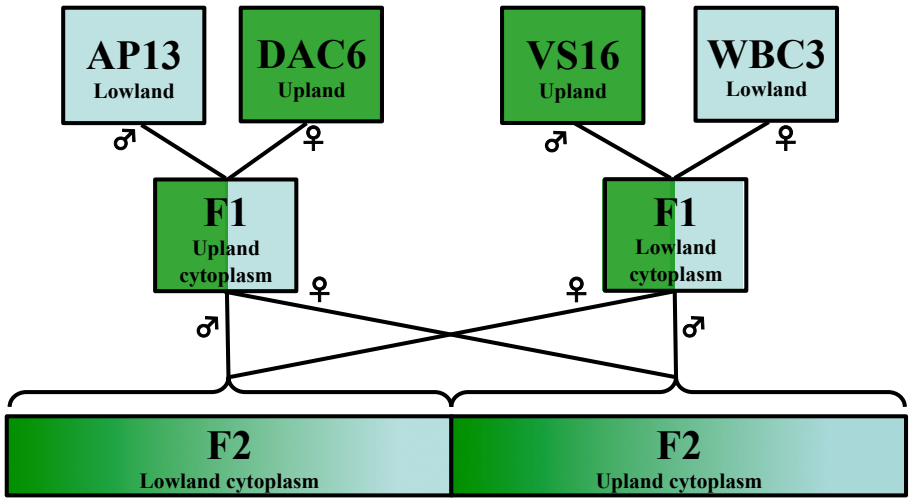
	Flower Date	Height	Tiller Mass	Tiller Number	Leaf Number	MD WP	SPAD	SLA
Height	0.265 ***							
Tiller Mass	0.232 ***	0.639 ***						
Tiller Number	0.157 ns	0.341 ***	0.165 *					
Leaf Number	0.066 ns	0.314 ***	0.408 ***	0.066 ns				
MD WP	0.216 **	0.09 ns	0.09 ns	0.064 ns	0.003 ns			
SPAD	0.101 ns	0.14 ns	0.151 ns	0.1 ns	-0.041 ns	0.084 ns		
SLA	-0.124 ns	-0.22 ***	-0.271 ***	0.065 ns	-0.199 **	-0.262 ***	-0.04 ns	
Rust PC1	0.149 ns	0.071 ns	-0.009 ns	-0.232 ***	-0.087 ns	0.221 ***	0.053 ns	-0.147 ns

Table 1. Spearman's rank correlation (rho) for phenotypic traits measured on the mapping population in the field. *, P<0.05; **, P<0.01; ***, P<0.001; ns, not significant. P-values corrected for multiple tests using the Holm-Bonferroni method. MD WP, midday water potential; SPAD, chlorophyll content; SLA, specific leaf area; Rust PC1, pathogen resistance

Phenotype	Linkage Group	Position (cM)	1.5 LOD CI (cM)	LOD	PVE	PDE	Effect Direction
Flowering Date	2K	113.28	106-122	9.13	9.1	74.09	+
Flowering Date	4K	83.41	54-86	8.77	8.72	70.99	-
Flowering Date	5K	98.4	86-172	5.32	5.17	42.09	+
Flowering Date	9N	132.87	16-158	5.09	4.94	40.22	-
Height*	2K	132.27	126.14-132.27	14.01	13.34	39.08	+
Height	3N	106.5	95.3-122	5.68	5.13	15.03	-
Height	5K	150	130-181.56	4.39	3.93	11.51	+
Height*	6N	61.39	56-65.35	13.32	12.63	37	-
Height	9N	130	120.45-142.6	5.81	5.25	15.38	-
Tiller Mass	2K	132	120-132.27	6.35	6.48	14.6	-
Tiller Mass	9K	24	10-49	6.55	6.69	15.07	+
Tiller Mass	2N	126	118-134	6.85	7.01	15.8	+
Tiller Mass	3N	114	96-124	7.45	7.65	17.24	+
Tiller Number	1N	70.15	59.25-79.25	7.1	8.19	85.8	+
Tiller Number	5K	98.4	86-136	4.46	5.06	53.01	+
Tiller Number	9K	83.86	76-94	4.43	5.02	52.59	-
Leaf Number	9K	42.37	30-48	6.36	8.25	80.82	+
SPAD	2K	113.28	96-123.5	5.67	7.43	97.94	-
Specific Leaf Area	5K	142.18	133.1-181.56	4.57	3.68	-19.66	-
Specific Leaf Area	5N	104.11	100-110	7.4	6.06	-32.37	+
Specific Leaf Area*	8N	24.38	22-30	13.87	11.85	-63.31	+
Specific Leaf Area*	9N	36.63	34.39-40	15.59	13.46	-71.91	+
Specific Leaf Area	9K	28	22-51.984	8.75	7.22	-38.57	+
Specific Leaf Area	9K	108	48.21-138	4.64	3.73	-19.93	+
Rust PC1	8K	13.21	6-52	4.86	6.22	25.91	+
GH Flowering Date*	2K	38.23	34-41.32	12	11.9	NA	-
GH Flowering Date*	5N	116.12	112-119.8	18.67	19.31	NA	+
GH Flowering Date	9K	98	52-114.79	4.31	4.08	NA	+
GH Height	3K	44	32.24-102.27	6.04	5.47	NA	+
GH Height	5N	119.8	110.13-122	6.08	5.5	NA	+
GH Height	7K	40.69	20-54	5.06	4.54	NA	+
GH Height	9N	102	70-130	4.79	4.3	NA	+
GH Height	9K	69.18	56-86	5.41	4.88	NA	+

Table 2. Significant QTL for traits measured in the field and in the greenhouse (GH). Each row represents a single QTL peak. *Trait**, epistatic interaction between QTL within trait; LOD, logarithm of odds; CI, confidence interval; cM, centimorgans; PVE, percent of phenotypic variance explained by QTL; PDE, percent of parental divergence explained by QTL; Effect direction (+), allelic effect consistent with ecotype divergence; Effect direction (-), allelic effect opposite of ecotype expectation.

747 **Figures**



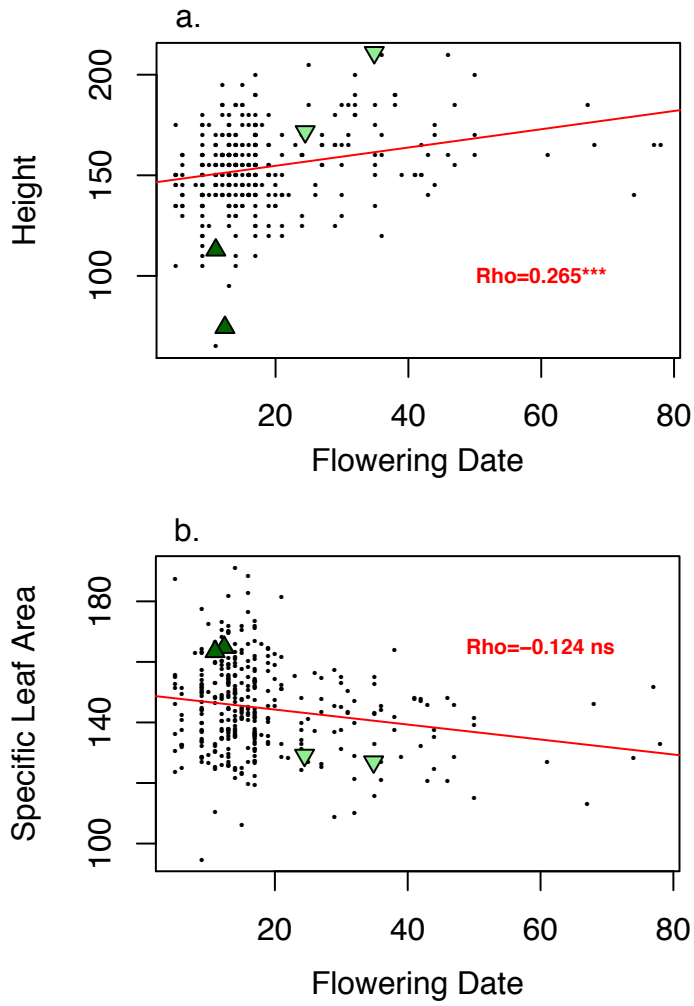
748

749

750 **Figure 1.** Diagram of four-way outbred reciprocal cross between two upland and two lowland

751 ecotypes of *P. virgatum*.

752

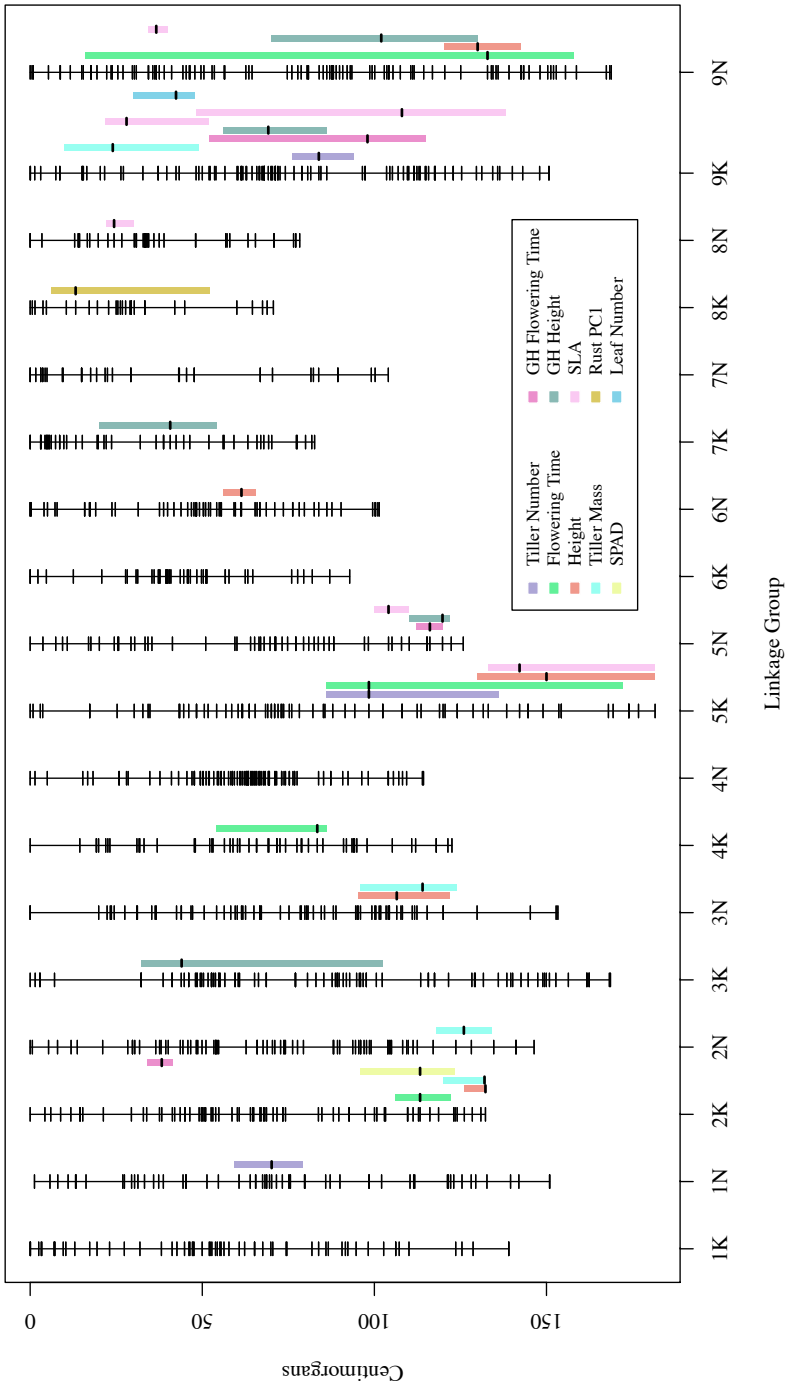


753

754 **Figure 2.** Sample scatterplots of traits correlations in the F_2 population with linear regression

755 line (red) and averaged trait values for the upland (dark green) and lowland (light green)

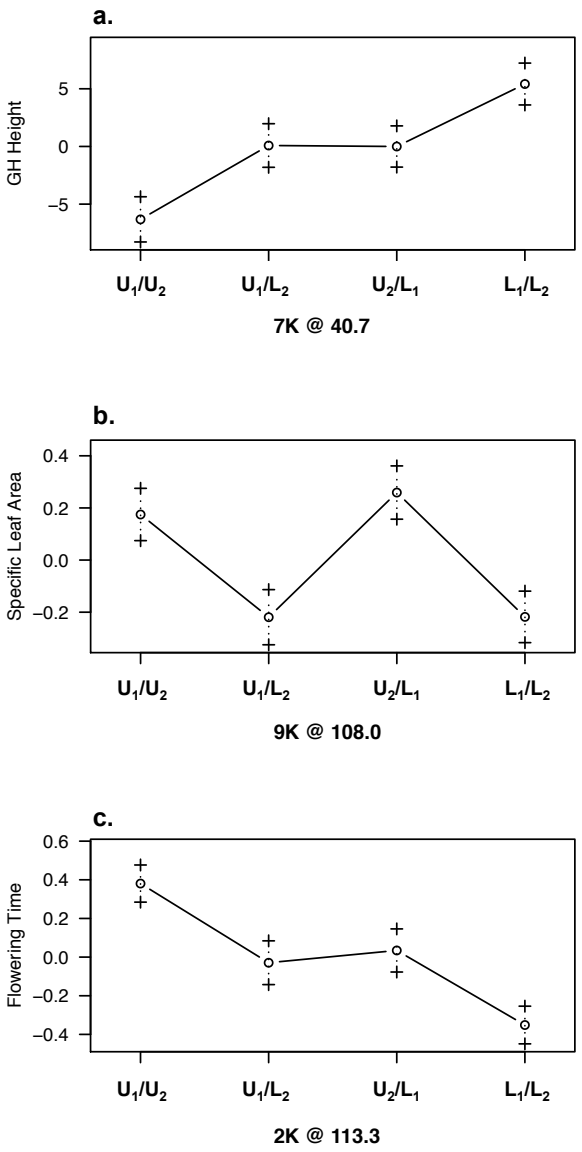
756 grandparent replicate plants.



757

758 **Figure 3.** Genetic linkage map for *P. virgatum* with QTL and 1.5 LOD drop confidence intervals

759 mapped to the right of their respective linkage groups.



760

761 **Figure 4.** Selected allelic effects plots to illustrate a) fixed ecotype effects, b) polymorphic

762 ecotype effects, and c) fixed ecotypic effect in the opposite direction of expected ecotype

763 divergence. The x-axis indicates genotype. U_1, DAC6; U_2, VS16; L_1, AP13; L_2, WBC3. Subtitle

764 indicates LG and marker position of the specific locus. GH, greenhouse traits.

765

Supporting Information

Figure S1. Photo of fungal pathogen infection.

Figure S2. Histograms of raw phenotypic values in the mapping population for all traits measured in the field.

File S1. Index and inline barcode sequences used to demultiplex raw sequence reads.

File S2. R/qtl file containing the joint genetic map and genotype and phenotype data for each individual in the QTL analysis.

File S3. Metadata for File S2 including phenotype descriptions and updated linkage group information.