Genetic Variation for Biomass Yield and Identification of Genomic Regions Associated with Regrowth Vigor and Salinity Tolerance in Lowland Switchgrass (*Panicum virgatum* L.)

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ABSTRACT

Switchgrass (*Panicum virgatum* L.) is a warm−season, perennial grass valued as a promising candidate species for biofuel feedstock production. The genetics underlying feedstock quality, stand stability under changing harvest frequency, and production of switchgrass on salt affected land is not well understood. Three studies were conducted to address these areas: (i) Genetic variation for biomass yield and predicted genetic gain in lowland switchgrass 'Kanlow', (ii) Assessment of genetic variation and identification of quantitative trait loci (QTL) associated with regrowth vigor in lowland switchgrass, and (iii) Identification of genomic regions associated with salinity tolerance in lowland switchgrass.

The results of the first study revealed significant genetic variation for biomass yield ($p < 0.05$) including feedstock quality traits hemi−cellulose ($p < 0.05$), and lignin ($p < 0.01$) content among Kanlow half−sib families. With a narrow sense heritability estimate of 0.10, a genetic gain of 16.5% is predicted for biomass yield in one cycle of selection using parental clones of 10% superior families. The second study utilized a nested association mapping (NAM) population to identify the genomic regions associated with regrowth vigor in switchgrass. The results showed notable variation among NAM families for regrowth vigor ($P < 0.05$). Ten QTL associated with regrowth vigor were detected, which accounted for phenotypic variation up to 4.7% and the additive genetic effects up to 0.26 unit of vigor score. In the third study, a subset of NAM population was used. A total of seven QTL associated with salt injury score (SIS) were identified, which accounted for phenotypic variation up to 6.5% and the additive genetic effects up to 0.63 unit of SIS. The transcript sequences of the identified QTL have a very high similarity with the genes found in closely related plant species of switchgrass. These genes are known to

play a variety of roles in the plant developmental processes and salinity tolerance mechanism, providing evidence that QTL could be useful in marker−assisted selection after validating their functional effect. The results of these studies would help breeders to integrate classical and molecular breeding to expedite switchgrass improvement.

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INTRODUCTION

Switchgrass

Switchgrass (*Panicum virgatum* L.) is a warm−season perennial grass of the family Poaceae which assimilates carbon via the C4 photosynthetic pathway. It is an important component of the North American pasture and tallgrass prairie. The natural habitat of switchgrass expands from Canada through the United States to Mexico, which indicates that it can adapt to a broad range of environments. Switchgrass accessions or cultivars are divided into two distinct ecotypes, lowland and upland, based on their natural habitat (Porter, 1966; Hultquist et al., 1996; Vogel, 2004; Casler, 2005). Lowland and upland ecotypes are also differentiated into either 'U' or 'L' cytotype based on chloroplast DNA polymorphism (Hultquist et al., 1997). The L cytotypes are tetraploids and associated with lowland ecotype, whereas, the U cytotypes are either tetraploids or octaploids and associated with upland ecotype (Hultquist et al., 1996). A few intermediate types, with an inconsistent chloroplast DNA polymorphism, are also reported which have possibly resulted from natural hybridization and gene flow between upland and lowland ecotypes (Hultquist et al., 1996; Zhang et al., 2011). Cytological study revealed various ploidy level among and within ecotypes that ranges from diploid (2n=2x=18) to dudecaploid (2n=2x=108) (Nielsen, 1944; Porter, 1966; Brunken and Estes, 1975; Anderson et al., 2016).

Upland ecotypes are characterized by thinner stems. Upland switchgrass is predominantly octoploid (2n=8x=72), but tetraploids (2n=4x=36) exist such as "Summer" (Barnett and Carver, 1967; Casler et al., 2011). Upland ecotypes are well adapted to dry environments and can tolerate cold temperature. In contrast, lowland ecotypes, are mainly identified by thicker stems, predominantly tetraploid genome (2n=4x=36) and adapted to relatively wet environments (Riley and Vogel, 1982; Hopkins et al., 1996; Casler et al., 2004; Vogel, 2004). "Alamo" and "Kanlow" are the most common examples of cultivars belonging to the tetraploid lowland ecotype group.

Phenotypically, lowland ecotypes are taller with coarser and larger leaf blades as compared to upland ecotypes (Vogel, 2004).

Historically, switchgrass has been utilized in prairie renovation, wildlife conservation, erosion control, and forage for livestock. It provides nutritious feed during mid−summer drought when most of the other forage species cease to grow. More recently, switchgrass has emerged as a model species for the development of cellulosic biomass feedstock. The perennial growth habit and the ability to produce high biomass yield in marginal lands with minimal fertilizer inputs have made switchgrass an ideal species for feedstock development (Vogel, 2004). In the 1990s the U.S. Department of Energy's Biofuels Feedstock Development Program selected switchgrass as a model herbaceous plant species for biofuel production, which led to intensive research towards improving switchgrass as a dedicated bioenergy crop (Vogel, 2004; Sanderson et al., 2006).

Switchgrass Floral Biology

It is necessary to understand floral biology and mode of reproduction of a species to determine breeding strategies (Acquaah, 2012). Switchgrass produces an inflorescence of about 15−55 cm long in the form of a diffuse panicle with a two−flowered spikelet which is 3−5 mm in length (Vogel, 2004). The florets are awnless and glabrous with upper floret being perfect and lower floret, either empty or staminate (Vogel, 2004). Switchgrass is predominantly an outcrossing species by virtue of a gametophytic self−incompatibility viz. the S−Z system (Talbert et al., 1983; Martínez−Reyna and Vogel, 2002; Casler et al., 2007; Casler, 2012). Cross−fertilization in switchgrass occurs between cytotypes with similar ploidy levels (Missaoui et al., 2006). Self−pollination is very rare in switchgrass; however, it has been reported that the rate of

selfing could be as high as 50% in some genotypes (Casler et al., 2011; Liu and Wu, 2012). Additionally, a post−fertilization incompatibility is also reported in switchgrass species which helps to eliminate the intercross among tetraploids and octaploids species (Martínez−Reyna and Vogel, 2002).

Switchgrass Breeding

Systematic breeding of switchgrass was started in the USA during the 1950s, and breeding efforts were primarily focused on germplasm collection, forage quality improvement, understanding reproductive biology, and breeding behavior (Vogel, 2004; Casler, 2012). Switchgrass germplasm collection and evaluation was initiated by plant materials centers (PMC) of the Soil Conservation Service (SCS), an agency of United States Department of Agriculture (USDA) which was later renamed the Natural Resource and Conservation Service (NRCS) (Vogel, 2004; Casler, 2012; Casler et al., 2015). The NRCS played a crucial role in releasing several switchgrass cultivars, including “Nebraska 28”, which became a first cultivar for which certified seed was produced (Vogel, 2004). There were remarkable breeding efforts before, but research has been strengthened since 1992 when the US Department of Energy (DOE) identified switchgrass as a model herbaceous feedstock (Sanderson et al., 2006). Commonly practiced conventional breeding methods used for the improvement of agronomic traits in switchgrass are: recurrent restricted phenotypic selection, half−sib progeny tests, among and within−family selection, and recurrent multi−step family selection (Vogel and Pedersen, 1993). These breeding methods capitalize additive genetic variation. Among all methods, half−sib family selection is the most frequently used breeding method. Several studies have been conducted in the past to understand genetics and apply the knowledge to improve biomass yield and agronomic traits by

capturing additive genetic variation in switchgrass (Vogel et al., 1996; Missaoui et al., 2005a; Casler, 2010, 2012). However, a few other studies demonstrated the potential for utilization of heterosis to increase biomass yield (Martinez−Reyna and Vogel, 2008; Vogel and Mitchell, 2008; Casler, 2014; Bhandari et al., 2017).

Areas of Genetic Improvement of Switchgrass

The genetic improvement of switchgrass can be categorized into two broad areas: livestock use and feedstock for biofuel production. However, specific breeding objectives and breeding efforts within either area are set forth by the individual breeding program according to the producer or consumer needs and environmental impact.

Genetic Improvement for Livestock Use

Forage yield and quality related to the daily live weight gain of an animal are crucial areas of research in switchgrass for livestock use. Past studies showed a significant improvement in forage quality measured as *in vitro* dry matter digestibility (IVDMD); however, it is negatively correlated with forage yield (Fritz et al., 1991; Vogel et al., 1991, 2013; Casler and Vogel, 1999). Therefore, forage yield without compromising quality, could be a vital cultivar breeding objective. Also, crude protein is an essential component of forage quality, but significant improvement has not been achieved (Vogel et al., 1984; Burns et al., 1985). A report published by Bates et al. (2012) suggests that the improvement of crude protein could be possible with proper harvest management. Additionally, a toxic chemical compound (Saponin) has been reported to be present in switchgrass which could cause a serious health problem in sheep (*Ovis aries*), horses (*Equus caballus*), and goats (*Capra aegagrus hircus*) (Lee et al., 2009). The presence of Saponins restricts switchgrass use as hay or pasture for these animals. In summary,

several opportunities exist for the improvement of forage quality along with forage yield in switchgrass.

Genetic Improvement for Biofuel Feedstock

Biomass Yield and Yield Components

Switchgrass as feedstock for biofuel production is a primary area of research for most breeding programs. Several studies have been conducted in the past to improve biomass yield and agronomic traits (Vogel et al., 1996; Missaoui et al., 2005a; Casler, 2010, 2012). These studies have suggested that biomass yield has been increased by 20 to 30% to date at the rate of 1−2% gain per year using recurrent selection (Casler, 2012). Genetic variation is very important in breeding as it provides the foundation for selection by which favorable alleles for a desired trait could be accumulated in the target population. A significant genetic variation was reported for biomass yield and yield−related components in different half−sib populations (Das et al., 2004; Rose IV et al., 2008; Bhandari et al., 2010). However, these studies were performed in space planted nursery with the plant to plant spacing ranging from 106 cm to 125 cm. Sykes et al. (2017) reported that studies conducted under space−plant nursery have low prediction power for biomass yield as compared to the densely planted nursery. A recent study conducted in moderately high density planted nursery (plant to plant spacing: 30 cm and row to row spacing 90 cm) validated the existence of a significant genetic variation among and within half−sib families (Dalid et al., 2018). Further, previous studies suggested that plant competition is essential to realize full expression of heterosis in switchgrass (Martinez-Reyna and Vogel, 2008; Vogel and Mitchell, 2008; Bhandari et al., 2017). All this information helps breeders to design appropriate breeding methods for directional improvement of biomass yield.

The success of a switchgrass cultivar largely depends on the consistent production of biomass for several years. Many studies indicated that lowland switchgrass is sensitive to frequent defoliation as compared to upland switchgrass (Neiland and Curtis, 1956; Beaty and Powell, 1976; Fike et al., 2006). Therefore, lowland switchgrass is conventionally harvested once per year after the killing−frost to achieve maximum yield. Research focused on improving the stability of biomass yield and regrowth vigor in lowland switchgrass under the changing harvest management could maximize switchgrass production.

A significant genotype × environment interaction (Casler, 2012) and genotype × plant Spacing interaction (Sykes et al., 2017) have been reported in switchgrass which complicates the breeder's job for making selection decisions. Often, breeders are interested in other traits such as plant height, stem thickness, tiller number, flowering date, and seedling vigor to make selection decisions for biomass yield as these morphological traits are reported to be correlated with biomass yield (Bhandari et al., 2010; Sykes et al., 2017).

Feedstock Quality

In addition to high biomass yield, continuous improvement of feedstock quality is vital for the success of switchgrass as a competent bioenergy crop. Lignocellulose is the main constituent of biomass dry matter, which has three major components: cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are the positive contributors of ethanol recovery by the biorefinery process, whereas lignin is an inhibitory substance that prevents the access of enzymes during the conversion process (Anex et al., 2007). However, lignin is an energy−rich compound with the energy content of approximately 25 MJ kg^{-1} and could be a useful source of energy required for ethanol distillation (Anex et al., 2007). Therefore, it is desirable to alter cellulose, hemicellulose, and lignin composition to improve the feedstock quality and overall ethanol

recovery. Composition of feedstocks may vary based on the growing region, genetic factors, morphology and agronomic practices (Mclaughlin et al., 1999; Vogel et al., 2002; Adler et al., 2006). Several studies in the past focused on improving forage quality (Vogel et al., 1984; Twidwell et al., 1988; Mitchell et al., 2001) as compared to feedstock quality. Therefore, it is necessary to accelerate the assessment and utilization of genetic variability of feedstock quality in switchgrass to improve ethanol recovery.

Host Plant Resistance to Insect Pests and Diseases

Several insects pests are reported to cause economic loss in switchgrass, especially in a monoculture setting (Prasifka and Gray, 2012; Torrez et al., 2013; Koch et al., 2014). Examples of some insects pests identified which cause significant damage in switchgrass are switchgrass moth (*Blastobasis repartella* Dietz) (Torrez et al., 2013), gall midge (*Chilophaga virgati* Gagne´) (Torrez et al., 2014), cereal aphid (*Sipha flava* Forbes) (Koch et al., 2014), and several species of grasshopper (*Melanoplus differentialis* Thomas; *M. bivittatus* Say; *Phoetaliotes nebrascensis* Thomas) (Chu and Knutson, 1970). Similarly, many pathogens are also reported to be a substantial threat for switchgrass cultivation for both pasture or feedstock use (Gravert and Munkvold, 2002). Switchgrass rust disease (caused by fungi *Puccinia emaculata* Schwein; *Puccinia virgata* Ellis & Everh; *Puccinia graminis* Pers: Pers; *Uromyces graminicola* Burrill), smut disease (caused by *Tilletia maclaganii*), anthracnose disease (caused by fungi *Collectotrichum navitas* sp. nov), and more recently leaf spot disease (caused by *Bipolaris oryzae* , teleomorph: *Cochliobolus miyabeanus*) are common diseases reported to reduce not only biomass but also the ethanol recovery (Gustafson et al., 2003; Krupinsky et al., 2004; Thomsen et al., 2008; Crouch et al., 2009; Sykes et al., 2016; Demers et al., 2017). Development of disease resistant or insect−pest resistant cultivar could be a potential area of research in

switchgrass. Abundant genetic variation exists among and within switchgrass populations related to host plant resistance. For example, Gustafson et al. (2003) described the existence of genetic variation for rust disease, which is an encouraging sign for a breeder to invest effort for developing rust−resistant cultivars through selection.

Abiotic Factors

Lowland cultivars are typically 30−50% more productive in biomass yield than upland cultivars; however, yield potential declines with the increase in latitude because of their inability to withstand prolonged winter in northern regions (Casler, 2012). It is reported that growing lowland switchgrass one hardiness zone north of their origin would cause a 9 to 17% reduction in biomass yield and survival (Casler et al., 2004). Improving cold hardiness in lowland switchgrass along with yield to adapt in the northern United States could be an important area of research in switchgrass (Casler and Vogel, 2014).

The premise that bioenergy feedstock should not compete with important field crops requires switchgrass cultivar development to be focused on their productivity under marginal lands. In general, abandoned or degraded croplands including saline, acid, drought or flood−prone, contaminated soils, highly erodible land, and reclaimed mine−soils are classified as marginal land (Blanco-Canqui, 2016). Drought and salinity are major contributors to marginal environments and have been progressively threatening modern agriculture system (Bartels and Sunkar, 2005; Hu and Schmidhalter, 2005; Mittler, 2006). Some studies have been done to understand morphological and physiological response of switchgrass in drought condition, to identify tolerant genotypes, and to develop strategies for battling severe moisture stress (Hu and Schmidhalter, 2005; Ghimire and Craven, 2011; Liu et al., 2015), These studies have reported some encouraging results to improve drought tolerance in switchgrass. On the other hand,

limited studies have been conducted to enhance salinity tolerance in switchgrass. A large proportion of agricultural lands have been identified as suffering from salinity issue across the globe. It is estimated that more than 800 million hectares of land across the world is suffering from salinity problem (Eynard et al., 2005; Anderson et al., 2015). In USA, over 5 million hectare of irrigated land (Eynard et al., 2005) out of 178 million hectare of arable cropland (Mclaughlin et al., 1999) is identified as having salinity problem. The level of salinity problems varies from place to place. Rhoades et al. (1999) categorized soil salinity based on the electrical conductivity (EC) of solute expressed in deci−Siemen meter^{-1} (dS m^{-1}): nonsaline (<2 dS m^{-1}), slightly saline (2–4 dS m^{-1}), moderately saline (4–8 dS m^{-1}), strongly saline (8–16 dS m^{-1}), and very strongly saline (>16 dS m^{-1}). Typically, EC of soil extract equivalent to 4 dS m^{-1} at 25°C, corresponds to approximately 40 mM concentration of NaCl (Hanin et al., 2016). High salinity could severely affect normal growth and development of plants, especially when genetic defense mechanism to counter this stress is not well developed. Munns and Tester (2008) reviewed that the mechanism of salinity tolerance in plants can be divided into three categories: tolerance to osmotic stress, Na^+ exclusion, and tissue tolerance. High salt concentration in the soil triggers osmotic stress to plants which causes an immediate reduction of cell expansion in root tips and young leaves and enforces stomatal closure. Plants which have the ability to tolerate a high level of osmotic stress can survive and continue to grow in the salty environment by maintaining a balance between water uptake by roots and stomatal conductance of leaves. Na^+ exerts toxic effects to plants when it accumulates to high levels in plants tissue. Na^+ is not considered an essential element but plays a crucial role in affecting enzyme activity in plants. High level of Na^+ can disrupt the various enzymatic process in the cytoplasm by competing with K^+, which is a crucial element for enzyme activation (Tester and Davenport, 2003). Thus, the two mechanisms

of salinity tolerance, Na^+ exclusion, and tissue tolerance, involve the ability of a plant to control Na^+ uptake by its roots or compartmentalize the excessive Na^+ at the cellular and intracellular level to safeguard the accumulation within leaves or the cytoplasm below the toxic level.

A few studies have been carried out in the past to understand genetic variation for biomass yield under salinity stress condition (Jakob et al., 2009; Kim et al., 2012; Anderson et al., 2015; Zhuo et al., 2015). Most of these were focused on learning about the effects of varying levels of salinity that a switchgrass plant can tolerate. Kim et al. (2012) reported that switchgrass plant could not survive when the salt concentration exceeds 400 mM. Anderson et al. (2015) found that switchgrass plant height was significantly affected by the increasing level of salinity. Also, Anderson et al. (2015) reported that lowland switchgrass cultivars EG 1101 and EG 1102 exhibited a high level of tolerance that tolerates salinity up to 10 dS m^{-1}. Greub et al. (1985) published that switchgrass showed severe injury to 2650 mM NaCl solution as compared to lemmon alkaligrass [*Puccinelliu lemmoni* (Vasey) Scribn.] and nuttall alkaligrass [*Puccinelliu uiroides* (Nutt.) Wats. and Coult.]. However, the development of switchgrass cultivar with a high level of salinity tolerance has never been prioritized. There is a tremendous opportunity for breeders to exploit available genetic variation for cultivar development in switchgrass with a high level of salinity tolerance.

Heritability of Trait of Interest

Most desirable agronomic traits are quantitative in nature, for example, yield. Knowledge of heritability helps determine the effectiveness of directional selection and improvement of such quantitative traits. Heritability gives us an estimate of the proportion of variance in a trait that is due to genetic effects. Genetic effects can be further partitioned into additive, dominance, and

epistatic effects. The proportion of phenotypic variance determined by additive genetic effect is referred to as narrow sense heritability. Additive variance is governed by additive genes which is fixable though selection; therefore, breeders are more interested in studying narrow sense heritability of a trait. Narrow sense heritability of several traits was estimated in the past including the biomass yield of switchgrass. These studies suggest that heritability of biomass yield tends to be low (Talbert et al., 1983; Bhandari et al., 2010; Edmé et al., 2017; Dalid et al., 2018). In such scenarios, breeders often look for the secondary trait which could be highly correlated to the trait of interest for use in indirect selection, such as plant height and tiller count for improving biomass yield (Das et al., 2004; Bhandari et al., 2010; Dalid et al., 2018).

Molecular Breeding and Biotechnology

Molecular breeding is the application of molecular tools to expedite the improvement of traits of interest or accelerate genetic gain. Molecular breeding includes marker−assisted selection (MAS), genomic selection (GS), and genetic modification (Allwright and Taylor, 2016). A recent development in genomic tools such as next−generation sequencing, high−throughput genotyping, and molecular breeding has opened a new avenue for molecular breeders to expedite the improvement of commercially important species (Allwright and Taylor, 2016). For example, genomic selection alone is expected to contribute three times the gain per year in biomass yield as compared to conventional breeding which requires arduous field evaluation for multiple years (Casler et al., 2011). Molecular−level study involves decoding of the information carried by genetic materials deoxyribonucleic acid (DNA). DNA based markers have been widely used in several crop species to study quantitative traits. The molecular breeding in switchgrass started with assessing the genetic diversity based on DNA content (Hultquist et al.,

1996; Casler et al., 2011). The study suggests that a base haploid genome size of octoploid switchgrass is 650 MB with total DNA weight of 5.2 picograms per nucleus, whereas a base haploid genome size of tetraploid species is approximately 750 MB with total DNA weight of 3.1 picograms per nucleus (Casler et al., 2011).

The nature of inheritance in tetraploid switchgrass is disomic, and the genome has 18 linkage groups distributed into two highly homologous sub−genomes of size approximately 1.5 GB (Okada et al., 2010; Casler et al., 2011). In switchgrass, restriction fragment length polymorphism (RFLP) markers have been used to create the first linkage map using biparental population AP13 × VS16 (Missaoui et al., 2005b). Progressively, a polymerase chain reaction (PCR) based markers have been developed such as simple sequence repeat (SSR), expressed sequence tag (EST)−SSR (Tobias et al., 2005, 2006, 2008), and genomic SSR (Wang et al., 2011). Okada et al. (2010), Liu et al. (2012), and Chang et al. (2016) used SSR and sequence−tagged−site (STS) markers to produce the genetic map. Single nucleotide polymorphism (SNP), the most abundant marker in the genome, has been commonly used to create ultra−high−density maps. Ali et al. (2019) published a high−density linkage map and identified QTL associated with growth traits in switchgrass using SNP markers generated through genotype-by-sequencing (GBS). The advancement of sequencing technology has greatly facilitated the genome−wide SNP discovery and genome−wide association study (GWAS). Lu et al. (2013) developed an association panel comprising 840 individuals and constructed ultra−high−density linkage maps containing a total of 88217 SNPs. Further, Childs et al. (2014) developed transcriptome (RNA−Seq) based SNPs, whereas, Evans et al. (2014) discovered exome−capture method to identify SNPs. Grabowski et al. (2016) conducted GWAS to understand the genetic regulation of flowering time in switchgrass using exome−capture sequencing data.

The development of molecular tools and in−depth knowledge of DNA sequence led to the development of a reference genome of lowland switchgrass genotype using AP13 (Alamo), which has been published by the Joint Genome Institute (http://www.phytozome.net). Most recently, genome editing, or CRISPR technology has attracted the attention of many breeders as it helps to edit the genetic architecture of the most valuable traits. In summary, available molecular tools and technology in conjunction with fundamental knowledge of plant breeding would increase the efficiency of the improvement of complex traits in switchgrass.

Objectives

This dissertation research was focused on studying genetic variation and detecting quantitative trait loci (QTL) for different traits in lowland switchgrass. The SNPs tightly linked with the identified QTL could be potentially utilized for marker−assisted breeding. The objectives of this dissertation research project are as follows:

1. To assess genetic variation for biomass yield and estimate genetic gain in lowland switchgrass
2. To assess genetic variation and identify genomic regions associated with regrowth vigor in lowland switchgrass
3. To identify genomic regions associated with salinity tolerance in lowland switchgrass

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CHAPTER 1

GENETIC VARIATION FOR BIOMASS YIELD AND PREDICTED GENETIC GAIN IN LOWLAND SWITCHGRASS 'KANLOW'

Abstract

Switchgrass (*Panicum virgatum* L.) is a warm−season, perennial grass native to the North American Prairie and widely recognized as an herbaceous bioenergy feedstock. Biomass yield is the most important trait for any bioenergy feedstock. Understanding the genetics underlying this trait is necessary to optimize the breeding method for full utilization of additive and non−additive genes. This study was focused on understanding the genetics underlying biomass yield and feedstock quality traits in a Kanlow population. The objectives of this study were (i) to assess genetic variation for biomass yield and the components of lignocelluloses, (ii) to estimate the heritability of biomass yield, and (iii) to examine if phenotypic selection provides any significant gain in biomass yield. Fifty−four Kanlow half−sib (KHS) families along with Kanlow check were planted in a randomized complete block design with three replications at two locations in Tennessee: Knoxville, and Crossville. The data were recorded for two consecutive years 2013 and 2014. The result suggested a significant genetic variation for biomass yield ($p < 0.05$), hemi−cellulose content ($p < 0.05$), and lignin content ($p < 0.01$). A significant KHS × location interaction ($p < 0.05$) in family performance was also observed. The narrow sense heritability estimates for biomass yield was very low (0.10) indicating a possible challenge to improve this trait. A genetic gain of 16.5% is predicted for biomass yield in each cycle of selection by recombining parental clones of 10% of superior progenies. Biomass yield in Kanlow can be improved by indirect selection based on taller plants and thicker stems because of moderate genetic correlation with plant height (0.54 ± 0.26) and stem thickness (0.75 ± 0.17). Improving biomass yield alone would lead to an increase in cellulose and lignin content in the Kanlow population.

Introduction

Switchgrass (*Panicum virgatum* L.) is a warm−season perennial grass native to the North American Prairie. Several uses of switchgrass have been reported which include forage for animals, soil stabilization for erosion control, and habitat for wildlife and migratory birds (Vogel et al., 2002; Casler et al., 2015). In the early 1990s, switchgrass was identified as model herbaceous energy crop by U.S. Department of Energy (USDOE) due to several desirable attributes including high biomass yield, relative ease to establish from seed, and harvest procedure (Mclaughlin et al., 1999; Vogel et al., 2002; McLaughlin and Kszos, 2005). This has encouraged scientists to conduct extensive research to improve switchgrass as a bioenergy crop. Renewable Fuel Standard (RFS−2) has mandated for the annual production of 21 billion gallons of advanced biofuel by 2022 (U.S. Department of Agriculture, 2010). This target could be achieved by cultivar breeding for improved biomass yield and lignocellulose component of a dedicated energy crop like switchgrass.

Tremendous genetic diversity exists for several traits within natural populations of switchgrass because of its out−crossing nature of reproduction mainly due to the S−Z system of gametophytic self−incompatibility (Martínez−Reyna and Vogel, 2002). As a result, the natural population are highly heterogeneous and heterozygous. Switchgrass has evolved in two distinct ecotypes – lowland and upland (Hultquist et al., 1996; Casler, 2005). Lowland ecotypes are spread across the southern region and adapted to a warmer and wetter climate, while upland ecotypes prevail in the northern region where the climate is relatively cold and dry (Mclaughlin et al., 1999; Vogel, 2004). A distinct morphological and cytological difference exists between these two ecotypes (Porter, 1966; Brunken and Estes, 1975; Riley and Vogel, 1982). Lowland ecotypes are characterized by having thicker−stems, taller, and high biomass yield as compared

to upland ecotypes. Based on chloroplast DNA polymorphism, switchgrass is also differentiated into either U or L cytotype (Hultquist et al., 1997). The L cytotypes are tetraploids and associated with lowland ecotype, whereas, the U cytotypes are either tetraploids or octaploids and associated with upland ecotype (Hultquist et al., 1996). A few intermediate types, with an inconsistent chloroplast DNA polymorphism, were also reported which has possibly resulted from natural hybridization and gene flow between upland and lowland ecotypes (Hultquist et al., 1996; Zhang et al., 2011). Cytological study revealed various ploidy levels among and within ecotypes that ranges from diploid (2n=2x=18) to dudecaploid (2n=2x=108) (Nielsen, 1944; Porter, 1966; Brunken and Estes, 1975; Anderson et al., 2016). However, most of the switchgrass cultivars are either tetraploid (2n=4x=36) or octaploid (2n=8x=72). Lowland ecotypes are tetraploid, while upland ecotypes are predominantly octaploid but a few tertraploids are also reported within upland ecotypes such as 'Summer' (Hultquist et al., 1996).

The success of switchgrass as feedstock for biofuel production highly depends on the improvement of biomass yield and its lignocelluloses composition such as cellulose, hemi−cellulose, and lignin. Cellulose and hemi−cellulose are the positive contributors of ethanol recovery by biorefinery process, whereas lignin is an inhibitory substance that prevents the access of enzymes during the conversion process. However, lignin is an energy−rich compound with the energy content of approximately 25 MJ kg^{-1} and could be a useful source of energy required for ethanol distillation (Anex et al., 2007). The advancement in biotechnology tools provides a promising route to genetically modify and expedite switchgrass cultivar development for high biomass yield and ethanol recovery; however, the regulatory requirements limit its scope at least for several years in the near future. Conventional breeding has historically played a significant role in improving the genetic architecture of biomass yield and other desirable traits

in important crops. Commonly practiced conventional breeding methods used in biomass yield improvement in outcrossing species such as switchgrass are: recurrent restricted phenotypic selection, half−sib progeny tests, among and within family selection, and recurrent multi−step family selection (Vogel and Pedersen, 1993). These breeding methods utilize additive genetic variation. However, the importance of heterosis has also been reported (Martinez−Reyna and Vogel, 2008; Vogel and Mitchell, 2008; Casler, 2014; Bhandari et al., 2017).

Several studies conducted in the past to improve biomass yield and agronomic traits in switchgrass were focused on capturing additive genetic variation (Vogel et al., 1996; Missaoui et al., 2005; Casler, 2010, 2012). Half−sib family selection is the most frequently used breeding method in switchgrass to capture additive genetic variation. A significant genetic variation has been reported for biomass yield and yield−related components in different half−sib populations (Das et al., 2004; Rose et al., 2008; Bhandari et al., 2010). These studies were performed in space planted nursery with a plant to plant spacing ranged from 106 cm to 125 cm. Sykes et al. (2017) reported that studies conducted under space−plant nursery have low prediction power for biomass yield as compared to the densely planted nursery. A recent study conducted in moderately high density planted nursery (plant to plant spacing: 30 cm and row to row spacing 90 cm) validated the existence of a significant genetic variation among and within Alamo half−sib families (Dalid et al., 2018). Predicted gain per cycle of selection for a trait depended upon the amount of genetic variation in the population, the heritability, the intensity of selection, and the efficiency of mating system (Vogel and Pedersen, 1993). The past studies suggest that biomass yield has been successfully increased by 20 to 30% to date at the rate of 1−2% gain per year using recurrent selection methods (Casler, 2012). The narrow sense heritability of biomass yield has been reported to be low (Talbert et al., 1983; Rose et al., 2008; Bhandari et al., 2010;

Dalid et al., 2018). Low heritability of biomass yield suggests a potential challenge to improve this trait. The narrow sense heritability of other agronomic traits such as plant height, stem thickness, tillering ability, and spring regrowth is reported to be moderately high to high (Talbert et al., 1983; Bhandari et al., 2010). On the other hand, the heritability of the major component of lignocellulose such as cellulose, hemi−cellulose, and lignin has not been extensively studied in switchgrass. Godshalk et al. (1988), Jahufer and Casler (2015), and Edmé et al. (2017) have reported heritability of feedstock quality traits to be moderately high based on family mean basis, however, the estimates based on individual plants are not available. An understanding of heritability of biomass yield and components of lignocellulose would be very helpful for breeders to predict the effectiveness of selection decisions.

A significant genotype × environment interaction has been well documented for biomass yield and other traits in switchgrass (Hopkins et al., 1995a; b; Casler and Boe, 2003; Casler et al., 2004). Lowland switchgrass produces high biomass yield; however, their survival becomes challenging in the northern region. It is reported that growing lowland switchgrass one hardiness zone north of their origin would cause a 9 to 17% reduction in biomass yield and survival (Casler et al., 2004). Improving cold hardiness in lowland switchgrass along with yield to adapt in the northern United States is an important objective of switchgrass breeding (Casler and Vogel, 2014). ‘Kanlow’, a high yielding lowland cultivar which was released in 1963 jointly by Kansas Agriculture Experiment Station (KAES) and the Agriculture Research Service (ARS)), has good adaptability from southern latitude to 40° N in North America and it can tolerate some extent of the cold environment as compared to high yielding lowland cultivar Alamo. Therefore, Kanlow has great potential to use in breeding for higher cold tolerance. In the past, most studies focused on studying genetic variation in lowland switchgrass have used Alamo, or Alamo derived

populations (Das et al., 2004; Missaoui et al., 2005; Rose et al., 2008; Bhandari et al., 2010; Dalid et al., 2018)**.** In contrast, not many studies were conducted on Kanlow, or Kanlow derived population except a few studies which utilized Kanlow genetic background to generate hybrid with upland cultivar 'Summer' (Edmé et al., 2017). This provides a good motivation to understand the extent of genetic variation in Kanlow and estimate genetic parameters such as heritability of important traits. The study reported here evaluated 54 half−sib families derived from Kanlow population, and the evaluation was carried out under moderately high plant−density. The objectives of this study were (i) to assess genetic variation for biomass yield and the components of lignocelluloses, (ii) to estimate the heritability of biomass yield, and (iii) to examine if phenotypic selection provides any significant gain in biomass yield in the Kanlow population.

Materials and Methods

Generation of Half−Sib Families

Kanlow half−sib (KHS) families of lowland switchgrass were generated in fall 2011. Over 200 genotypes were selected from a four−year−old sward of Kanlow switchgrass which was established in Fall 2007 at Holston Unit Farm of the University of Tennessee, East Tennessee Research and Education Center (ETREC) (35°58'32" N; 83°51'25" W). Individual plant selection was based on visual vigor at maturity. Open pollinated seeds were harvested separately from each of the selected plants. Collected seeds were threshed, cleaned, and stored separately in envelops. The KHS families which had enough seeds (approximately 200 seeds) were retained and advanced to germination. To break seed dormancy, seeds were treated with 100% household bleach (5.25% Sodium Hypochlorite) for 15 minutes, rinsed twice with tap

water, and wet−chilled at 4 °C for one week (Bhandari et al., 2010). Treated seeds were placed for germination on wet filter paper in the Petri−dishes and incubated at room temperature (24 °C). Germinated seedlings of KHS families were transplanted in 72−well flats filled with greenhouse soil (Metromix 300, Griffin Greenhouse) and seedlings were raised in the greenhouse (25/15 °C day/night; 16h light) for approximately 12 weeks. Finally, fifty−four KHS families which satisfied the number of seedlings required for replicated field testing were used in the experiment.

Field Evaluation

The KHS families were evaluated in the field at two locations in Tennessee, Holston Unit Farm of ETREC, Knoxville (35°58'42" N; 83°51'28" W), and Plateau Research and Education Center (PREC), Crossville (36°00'56.3" N; 85°07'56.0" W). Soil type in the Knoxville was Shady−Whitwell complex (fine−loamy, mixed, subactive, thermic Typic Hapludults; fine−loamy, siliceous, semiactive, thermic Aquic Hapludults), and at Crossville was Lonewood loam (fine−loamy, siliceous, semiactive, mesic Typic Hapludults). The experiment was conducted in a randomized complete block design with three replications at both locations. Seedlings of 54 KHS and Kanlow original population as check were transplanted in the field nursery at ETREC and PREC on May 31 and June 7, 2012, respectively. Each family in each replicate was planted in a 9−plant single−row plot, with the plant to plant spacing 0.3 m, and row to row spacing 0.9 m. Fertilizer was not applied during the establishment years. The plot was amended with 60 Kg ha^{-1} N each spring during the post−establishment year. To keep weed pressure minimum, pre−emergence herbicide Dual II Magnum (Metolachlor; Syngenta Crop Protection, Inc., Greensboro, NC) at the rate of 2.84 L ha^{-1}, and Prowl H_2O (Perdamethalin; BASF Corporation, Research Triangle Park, NC) at the rate of 3.31 L ha^{-1}; and post−emergence application of 2,4−D

at the rate of 2.37 L ha^{-1} with surfactant at the rate of 1.18 L ha^{-1}, were applied after a week of transplanting and during early spring in each post−establishment year. Holston Unit Farm, ETREC, Knoxville plots had been severely infested with Nutsedge (*Cyperus* Spp.) during early growth stage, therefore, a post−emergence herbicide Accent (DuPont) at the rate of 18.9 g ha^{-1} mixed with crop oil (1% v/v) was applied approximately 60 days after transplanting.

Data Collection

No data were recorded during the establishment year (2012). The plots at both locations were mowed at the end of the growing season. Biomass yield data for 2013 growth was recorded on Nov 14, and 19 at Crossville and Knoxville respectively; while for 2014, biomass was harvested on Dec 14 at Crossville and on Jan 22, 2015, at Knoxville. In 2013, five tillers were randomly sampled from five plants in each plot to estimate within family variation. The tiller samples of individual genotype were dried and weighed separately. Other phenotypic data such as plant height at maturity was recorded in centimeters; only five plants per plot from 2 replications were measured. Stem thickness (1 = the smallest to 5 = the largest stem thickness), and tillering ability (1 = less than 10 tillers to 9 = more than 80 tillers) were also recorded at the same time from the same plants at both locations.

Feedstock Composition Analysis

Above ground plant tillers were collected just before biomass harvesting. Five tillers were randomly collected from each of five plants of a plot in two replications. Sampling was done individually in 2013. However, 2014 composition analysis samples were collected in bulk at the time of harvesting. Samples were oven dried and ground in a two−step process; coarse ground using Wiley Mill (Thomas Scientific, Swedesboro, NJ) and finely ground using a Cyclone grinder (UDY Corp., Fort Collins, CO) to pass through 1 mm mesh. Ground samples

were scanned using SpectraStar^{TM} Unity Scientific near−infrared spectroscopy (NIRS) platform to estimate the components of lignocelluloses – cellulose, hemi−cellulose, and lignin content. Cellulose content was calculated by subtracting lignin from ADF (Acid Detergent Fiber), whereas, hemi−cellulose content was computed by subtracting ADF from NDF (Neutral detergent fiber) (Hopkins et al., 1995a; Casler and Vogel, 2014).

Data Analysis

Variance Component

Data were analyzed using the MIXED model analysis (PROC MIXED) in SAS 9.4 (SAS Institute, Cary, NC). Variance components for each trait were estimated using the restricted maximum likelihood (REML) method. In the data analysis model, location and year were considered fixed, whereas, replication and family were considered random. Plot mean data were calculated for each year (2013 and 2014) and location (Knoxville and Crossville) to analyze genetic variation among KHS families. Within KHS family variation of biomass yield was calculated for the year 2013 using individual plant biomass data. Biomass weight of individual plant within a family plot was calculated by multiplying average tiller weight with total tiller count.

Comparison of Mean Performance of Traits

Biomass yield and other trait data were analyzed using the MIXED model analysis in JMP Pro 14 (SAS Institute, Cary, NC). In the mixed model analysis KHS, location, and year were considered fixed effects factors and replication was considered a random effect factor. Least square means were obtained for each KHS family and Kanlow Check. Any significant difference between the least square means was detected using Fisher's protected LSD ($p < 0.05$). The efficiency of phenotypic selection was tested by comparing group means of KHS and

Kanlow check using an orthogonal contrast. Box plots to visualize biomass yield of KHS families were generated using SAS Enterprise Guide 7.1(SAS Institute, Cary, NC).

Narrow Sense Heritability

Narrow sense heritability of biomass yield, the components of lignocellulose, and other morphological traits were estimated on an individual plant basis by using variance components that were obtained from the analysis of variance. The equation used to compute the narrow sense heritability was based on equation described by Eberhart and Newell (1959):

$$h^2 = \frac{\sigma_A^2}{\sigma_P^2} = \frac{4 \times \sigma_{hs}^2}{\sigma_{hs}^2 + \sigma_{hs \times Y}^2 + \sigma_{hs \times L}^2 + \sigma_{hs \times Y \times L}^2 + \sigma_w^2}$$

where,

$\sigma_A^2 =$ additive genetic variance

$\sigma_P^2 =$ phenotypic variance

$\sigma_{hs}^2 = \frac{1}{4} \times \sigma_A^2 =$ variance among half−sib families

$\sigma_{hs \times Y}^2 =$ vaiance due to family $\times$ year interaction

$\sigma_{hs \times L}^2 =$ vaiance due to family $\times$ location interaction

$\sigma_{hs \times Y \times L}^2 =$ vaiance due to family $\times$ year $\times$ location interaction

$\sigma_w^2 =$ variance associated with the difference in plants within a half − sib family

Phenotypic and Genetic Correlation

The phenotypic and genetic (additive) correlation were computed in SAS by using Proc MIXED restricted maximum likelihood (REML) method as described by Holland (2006). The estimation of the phenotypic and genetic correlation was based on the equation described by Miller et al. (1958).

$$\text{Genetic correlation } (r_g) = \frac{\sigma_{g(\text{trait 1,trait 2})}}{\sqrt{\sigma^2_{g(\text{trait 1})} \times \sigma^2_{g(\text{trait2})}}}$$

$$\text{Phenotypic correlation } (r_p) = \frac{\sigma_{p(\text{trait 1,trait 2})}}{\sqrt{\sigma^2_{p(\text{trait 1})} \times \sigma^2_{p(\text{trait2})}}}$$

Where,

$\sigma_{g(\text{trait 1,trait 2})}$ = the genetic covariance between trait 1 and trait 2

$\sigma_{p(\text{trait 1,trait 2})}$ = the phenotypic covariance between trait 1 and trait 2

$\sigma^2_{g(\text{trait 1})}$ = the genetic variance of trait 1

$\sigma^2_{g(\text{trait2})}$ = the genetic variance of trait 2

$\sigma^2_{p(\text{trait 1})}$ = the phenotypic variance of trait 1

$\sigma^2_{p(\text{trait2})}$ = the phenotypic variance of trait 2

Predicted per Cycle Genetic Gain

Predicted per cycle genetic gain (ΔG) from selection were calculated using the equation described by Nguyen and Sleper (1983).

$\Delta G = k\ c\ h^2\ \sigma_p$

where,

k = the standardized selection differential (for 10%, *k* = 1.76: for 15%, *k*=1.55)

c = parental control factor (for remnant seed, *c* = 1; for parental clone, *c* = 2)

h^2 = narrow sense heritability on an individual plant basis

σ_p = phenotypic standard deviation

Results and Discussion

Variance Components of Biomass Yield, Morphological, and Quality Traits

Variance components of biomass yield are presented in Table 1−1. A significant genetic variation ($p < 0.05$) among KHS families was observed for biomass yield across locations (Knoxville and Crossville) and years (2013 and 2014). A total of 9.7% of phenotypic variation among KHS families was contributed by the additive genetic effects for the combined data. Genetic variation for biomass yield among KHS was found to be broader within each location when analyzed separately ($p < 0.01$, Table 1−1, Figure 1−1). The additive genetic variation for biomass yield accounted for 22% and 19% of the total phenotypic variation of KHS families in Knoxville and Crossville, respectively. Our result showed that a large amount of variation was shared by the plants within−family variation. Theoretically, half−sib family accounts for one−fourth of the total additive genetic variance, whereas three−fourth of additive plus dominant variance is expected to be present within half−sib families (Hallauer et al., 1988). On the basis this theoretical background, our results for among and within KHS family variation indicate that additive genes played an important role in biomass yield, but non-additive genes might be abundant. Although, we could not separate the additive and dominance variance present within half−sib families because of the half−sib mating system used in this study unlike the nested mating design used by Bhandari et al. (2011).

Biomass yield was influenced by KHS × location interaction ($p < 0.05$) (Table 1−1, Figure 1−2). KHS × location interaction was possibly due to the varying response of KHS family to the difference in temperature and precipitation. The two experiment sites located within similar latitude, but elevation difference likely contributed to the temperature difference. Crossville is located at an elevation of 580 m, as compared with 270 m for Knoxville. On

average, the temperature in Crossville tends to be cooler than Knoxville. During the experiment period, the average annual temperature of Crossville was recorded to be 2.9 °C lower than Knoxville (based on the climate data obtained from (https://www.ncdc.noaa.gov). Casler et al. (2004) reported that temperature and photoperiod are important factors in determining switchgrass adaptation. This is likely due to the fact that the enzymatic activity linked to the metabolic pathways such as photosynthesis, and respiration are affected by temperature fluctuation which ultimately influences normal growth and development of switchgrass plants. A good example of temperature effect on switchgrass is that onset of winter (killing frost) arrests growth and imposes dormancy in the rhizomes (Sarath et al., 2014). Subsequently, the rise of temperature in the following spring helps to break dormancy and drives above-ground growth. However, if the genotype is sensitive to low temperature, spring frost could push back the regrowth in early breaking genotypes. Genotypes having some extent of cold tolerance would continue to grow as enzymatic activity would not be affected by reduced temperature during spring. Our results revealed that some families are less affected by the difference in temperature and produce consistently higher biomass across location (Figure 1−1). Additionally, Crossville received higher precipitation (128.7 mm) than Knoxville (116.2 mm) during the experiment period from 2012 to 2014. The amount of precipitation received at Knoxville and Crossville was even more inconsistent during the active growing season (April to September) of switchgrass. During the establishment year (2012), Crossville received 25.4 mm less precipitation than Knoxville. Conversely. in 2013 and 2014, Crosville received 15.9 mm and 47.5 mm more precipitation than Knoxville. Hui et al. (2018) reported that increased precipitation significantly enhances biomass of switchgrass by stimulating leaf photosynthesis. We observed a yield gap of above ground plant biomass of KHS family between Knoxville and Crossville increased to 8.3 t

ha^{-1} in 2014 than 5.3 t ha^{-1} in 2013 (data not presented). Based on this evidence, we speculate that variation in precipitation could have affected biomass accumulation in some switchgrass family by interfering with photosynthetic activity. Genotype × location interaction was such a high component in the current study that the biomass yield at the Knoxville explained only 10% of the variation in biomass yield at the Crossville (Figure 1−2). Our result is in agreement with previous studies for a genotype × location interaction (Hopkins et al., 1995b; Bhandari et al., 2010), validating the necessity of multi−location experiments for making selection decisions to improve biomass yield in switchgrass. Also, the existence of genotype × location interaction emphasizes the development of regionally adapted cultivars to maximize genetic gain in the target region (Casler et al., 2007; Casler, 2012).

KHS interaction with year and location × year were not evident in this study for biomass yield. The fixed effect of locations ($p < 0.01$), year ($p < 0.01$), and location × year interactions ($p < 0.01$) were highly significant. The magnitude of year effect was the largest ($p < 0.01$) on biomass yield variation, which is not surprising for perennial grasses like switchgrass. It is well documented that switchgrass reaches to full yield potential only by the third year of growth because maximum energy is diverted for root development during establishment years (Mclaughlin et al., 1999).

For morphological and feedstock quality traits measured in this study, there was no significant variation observed for plant height, tillering ability, stem thickness, and cellulose percentage among KHS families, however, a notable difference was observed in hemi−cellulose ($p < 0.05$) and lignin ($p < 0.01$) percentage (Table 1−2). Stem thickness showed KHS × year interaction effect ($p < 0.01$). No other morphological and feedstock quality traits showed KHS × year or KHS × location interaction. For all measured traits, substantial variation was attributed to

the plants within KHS ($p < 0.01$) which is confounded with random error variation. Within family variation could potentially mislead the true genotypic variation and selection decision. One way to improve precision of estimating error variance would be by utilizing clonal copies of genotypes in the experiment. Casler and Brummer (2008) specified clonal replication is not widely adopted by breeding programs because of time and other resource burdens involved with it. However, a breeder could invest resources for propagating the clonal copies of genotypes, for example tissue culture or other feasible methods, that would make it possible to estimate error variance. Estimation of error variance would lead to understanding of the genetic effects more precisely. It is advised to use at least one clonal copy that would represent true replication of the genotype in the experiment.

The fixed year effects were highly significant ($p < 0.01$) for all measured traits except tillering ability, whereas, the location effect was only evident for feedstock quality traits− cellulose ($p < 0.01$), hemi−cellulose ($p < 0.05$), and lignin ($p < 0.01$). All measured traits, except tillering ability, were influenced by the location × year interaction effects.

Summary statistics of biomass yield and other measured traits are presented in Table 1−3. Biomass yield of KHS family at Knoxville ranged from 2.2 t ha^{-1} to 18.5 t ha^{-1} with the mean value of 9.5 t ha^{-1}. At the Crossville location, biomass yield ranged from 4.4 t ha^{-1} to 30.4 t ha^{-1} with the mean value of 17.3 t ha^{-1}. Mean biomass yield of KHS family across locations (Knoxville and Crossville) and years (2013 and 2014) ranged from 9.6 t ha^{-1} to 16.9 t ha^{-1} with the mean value of 13.4 t ha^{-1}. Nine KHS families produced higher biomass as compared to Kanlow check ($p < 0.05$) (data not presented). Biomass yield of 42 KHS families did not show any statistical difference than Kanlow check, whereas, 3 KHS families produced significantly lower biomass yield.

Above ground height measured for KHS families ranged from 226 cm to 262 cm (Table 1−3). The KHS family height was neither taller nor shorter than Kanlow check. KHS families stem thickness score ranged between 3 to 4.2, and only nine families had thicker stem than Kanlow check, but none had a thinner stem. Mean tillering ability score of the KHS families ranged from 2.9 to 4.5, but none of the families had a significantly different score than Kanlow check.

Mean value of feedstock quality traits−cellulose, hemi−cellulose, and lignin were 44.6, 33.7, and 6.3, respectively (Table 1−3). Cellulose percentage ranged from 43.9 to 45.4, of which only two families had superior, but none had an inferior composition of cellulose compared to Kanlow Check. Hemi−cellulose percentage ranged between 32.6 to 35.1. One KHS family had superior hemi−cellulose percentage, two families had inferior, and all other families were not statistically different. Lignin percentage ranged from 5.6 to 6.9. Six families composed higher lignin percentage, but the rest of the families had a statistically similar composition to Kanlow check. The results suggest that improvement of cellulose and hemi−cellulose is possible through among or within family selection. Depending upon the use of feedstock, lignin content could also be altered in Kanlow population via classical breeding.

Efficiency of Phenotypic Selection

In this study, we used open−pollinated seeds that were harvested from the most vigorous plants in four−year−sward of Kanlow switchgrass. The plants were identified based on visual vigor and overall phenotypic appearance at maturity. The performance of selected progenies was compared with the check to see if phenotypic selection offered any gain. For phenotypic selection to be effective, we expected that KHS would produce higher biomass yield as compared to Kanlow check. Our result suggested that KHS did not produce higher biomass yield

(13.4 t ha^{-1}) as compared to Kanlow check (12.9 t ha^{-1}) (Table 1−3, Figure 1−3). The result suggested that phenotypic selection was not efficient to change the population mean in Kanlow. Our result is consistent with previous studies conducted in an Alamo derived population. (Bhandari et al., 2013; Dalid et al., 2018).

The phenotypic selection was not effective in our study suggesting that selection solely based on individual plants fitness would not be efficient for the complex traits like biomass yield. The individual genotypes variation in sward could have been associated with environmental variation such as topography, soil texture, soil nutritional status and another microenvironment within sward. In this study, morphological traits such as plant height, stem thickness, and tillering ability showed a wide range of distribution within each location. However, the range of these traits was drastically narrowed when mean data across locations and years were analyzed. Genetic variation for biomass yield was observed, but within−family variation was very high leading to low heritability. Brown et al. (2014) suggests that phenotypic selection would not be efficient for the traits having low heritability. Further, Falconer and Mackay (1996) described that pollen from undesirable plants could dilute the desirable alleles in the selected plant. Planting of the selected plant into an isolated polycross block to generate plant materials could produce favorable recombinants. However, this process would significantly delay the selection cycle, thus reducing genetic gain per year. Casler (1999) recapped that most field-based phenotypic selection in forage crops require 2 year per cycle. If phenotypic selection is the breeder's interest, then it could be performed by applying some restriction as practiced by Burton (1974) in Pensacola bahiagrass (*Paspalum notatum* var. *saure* 'Parodi').

Heritability

Estimates of narrow sense heritability provides a foundation to predict genetic progress that could be achieved through the breeding cycle. Out of seven traits considered in this study, only biomass yield, hemi−cellulose, and lignin showed the presence of genetic variation to make it possible to estimate heritability. The estimates of narrow sense heritability based on variance components are presented in Table 1−4. The estimates of narrow sense heritability for biomass yield, hemi−cellulose, and lignin were 0.10, 0.32 and 0.66, respectively. The estimate was very low for biomass yield because the computation was done on an individual plant basis. Low heritability on an individual plant basis was observed due to the fact that the quantitative traits measured on individual plant produced a large residual effect. Previous studies conducted using Alamo derived population reported a similar estimate of heritability for biomass yield (Talbert et al., 1983; Rose et al., 2008; Bhandari et al., 2010; Dalid et al., 2018). The fact that narrow sense heritability is a function of additive variance, genotype × environment interaction, and random error, changes in the magnitude of any of these variances would alter heritability. Low heritability of biomass yield in this study could be associated with a very high genotype × environment interaction and random error. This is reflected in the higher heritability estimates within each location (Knoxville h^2 =0.22 and Crossville, h^2 = 0.19). Low heritability estimates for biomass yield hinted for possible challenges to improve this trait. For difficult traits like biomass yield, selection based on a secondary trait which is highly heritable and correlated with the trait of interest, could be very effective to make positive progress.

Genetic and Phenotypic Correlation

Breeders often rely on indirect selection if primary traits of interest are very difficult or expensive to measure because of several factors such as polygenic nature, low heritability,

and the presence of genotype × environment interaction (Falconer and Mackay, 1996). Success of indirect selection relies on the high heritability of the secondary trait, easiness of selection, and correlation with the primary trait. Therefore, knowledge of phenotypic and genetic correlation among different traits are important in plant breeding. A phenotypic correlation between two traits illustrate the correlation of both genetic and environmental effects. The genetic correlation, on the other hand, indicates that the genes that contribute to the traits are usually co-inherited, and is more important to the breeder for improving difficult traits through indirect selection. The genetic and phenotypic correlations of biomass yield with plant height, stem thickness, tillering ability, cellulose, hemi−cellulose, and lignin, are presented in Table 1−5.

Results showed biomass yield had positive phenotypic correlation with plant height (0.27 ± 0.06), stem thickness (0.31 ± 0.06), and lignin (0.15 ± 0.06); low but positive correlation with tillering ability (0.19 ± 0.05) and cellulose (0.16 ± 0.05); and very low negative correlation with hemi−cellulose (-0.08 ± 0.05). Bhandari et al. (2010) and Sykes et al. (2017) reported a stronger positive correlation of biomass yield with plant height and stem thickness twice as much as the phenotypic correlation we found in this study. Also, tillering ability was found to have a poor correlation with biomass yield in our study contradicting the result published by Das et al. (2004). But as mentioned earlier, it should be noted that their results were based on the study conducted in a space planted nursery which has low prediction power as compared to the densely planted nursery.

Genetic correlation of biomass yield with plant height (0.54 ± 0.26) and stem thickness (0.75 ± 0.17) was moderate to moderately strong in the present study, suggesting that the combination of these two traits have potential to serve as the good candidates for indirect selection to improve biomass yield. However, breeders need to be vigilant as we were not able to

estimate heritability of neither plant height nor stem thickness in our study because of non−significant genetic variation among KHS family. We assume that non−significant genetic variation of these two traits could be associated with type I error due to small population size in this study which may not be the case if a large population would have been used. For example, Jahufer and Casler (2015) reported an increment in heritability estimate for biomass yield using a bigger population size. Past studies in Alamo derived population presented moderate heritability for plant height and stem thickness (Bhandari et al., 2010, 2011).

Among quality traits, we observed a positive genetic correlation of biomass yield with cellulose (0.39 ± 0.28) and lignin (0.44 ± 0.20). Positive genetic correlation between biomass yield an lignin in our study is in agreement with Jahufer and Casler (2015). Conversely, Edmé et al. (2017) reported negative correlation between biomass yield and lignin in their studies (-0.33 ± 0.22). The negative correlation between biomass yield and lignin, as reported by Edmé et al. (2017), may be a result of the upland switchgrass genetic background involved in their study. The most desirable feedstock quality trait (cellulose) had a very strong positive genetic correlation with plant height (0.90 ± 0.42) and stem thickness (0.98 ± 0.33). Plant height and stem thickness were also positively correlated with biomass yield. Among traits considered in this study, stem thickness showed the strongest genetic correlation with both biomass yield (0.75 ± 0.17) and cellulose (0.98 ± 0.33). Based on these results, it is evident that improving biomass yield alone would lead to an increase in cellulose and lignin content in the Kanlow population. To make the feedstock industry more profitable, the ideal breeding procedure in switchgrass is to improve biomass yield in parallel with improving the quality traits. Depending upon the use of biomass feedstock i.e for ethanol recovery, pyrolysis, or combustion, breeders could have opportunity to modify cellulose and lignin by improving biomass yield via. classical breeding.

Improvement of biomass yield in conjunction with high cellulose or lignin content in Kanlow can be achieved by indirect selection based on taller plants and thicker stems; however, the breeder needs to look for additional traits (individual or combination of two or more traits) in future studies such as “leafy tillers”, “plant posture”, “thicker stem with high tillering ability” or “thicker stem with high leaf number” that would potentially have a high positive correlation with biomass yield and feedstock quality traits.

Predicted per Cycle Genetic Gain

Using the heritability computed in this study, we predicted selection gain for biomass yield (Table 1−6). With selection intensity of 15% and using remnant seeds of selected half−sib families (PC=1), a gain of biomass yield is predicted to be 0.97 t ha^{-1}(7.2%). Using remnant seed and applying stringent selection pressure, i.e. 10%, a gain of biomass yield is predicted to be 1.10 t ha^{-1} (8.2%). While with the selection intensity of 15% but using parental−clones of selected half−sib families as a recombination unit, biomass yield can be doubled (a gain of 1.95 t ha^{-1} or 14.6%). The gain could be further enhanced when recombining only 10% of superior parental clones (a gain of 2.21 t ha^{-1} or 16.5%).

Similarly, with a selection intensity of 15%, hemi−cellulose content could be improved by 2% using remnant seeds or 4% by using parental−clones of selected half−sib families. With selection intensity of 10%, hemi−cellulose content could be enhanced by 2.2% using remnant seeds or 4.4% by using parental−clones of selected half−sib families. The result suggests that maximum genetic gain is possible for both biomass yield and hemi−cellulose by recombining clonal parents of 10% superior families.

Conclusion

Development of high yielding switchgrass cultivars along with better feedstock quality and a wide range of adaptability is necessary to sustain future bioenergy requirements. It is necessary to adopt an ideal breeding method for full utilization of additive and non−additive genes. The results of the current study showed the existence of genetic variability for biomass yield both among and within Kanlow population. Selection based on single plant performance may not be effective to improve biomass yield. Accumulation of favorable additive genes to improve biomass yield could be practiced by employing rigorous family−performance−based selection or among−and−within family selection (Casler and Brummer, 2008). The results also signify that genes controlling biomass yield are highly influenced by genotype × location interaction, suggesting the necessity of a target region based multi−location experiment for making the selection decision. Indirect selection based on morphological traits such as stem thickness may improve both biomass yield and feedstock quality. Maximum genetic gain can be achieved by recombining parental clones of the top 10% of superior families.

The magnitude of half−sib family variance is much smaller than within−family variance, and heritability is low for biomass yield which provides a hint that non−additive genes are abundant illustrating the scope of heterosis breeding. Currently, the genetic gain achieved by switchgrass breeders mostly relies on additive genes. Further study using a different mating design, such as diallel or nested design (North Carolina Design I), would be helpful for partitioning additive and dominance variance. Previous studies demonstrated a successful utilization of heterosis breeding utilizing ‘Kanlow × Summer’ and ‘Alamo × Kanlow’ cross (Martinez-Reyna and Vogel, 2008; Casler and Vogel, 2014; Bhandari et al., 2017). Kanlow is a frequently used parent source in heterosis breeding programs, likely due to its ability to adapt in

both southern region and a narrow strip of the northern region. Lack of synchronization in flowering time between Kanlow with counterpart heterotic group could serve as a barrier for hybrid seed production in large scale. In the future, studies focused on heterosis breeding along with flowering time synchronization with counterpart heterotic group may help to intensify biomass production.

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Appendix I

Table 1–1. Variance components and tests of fixed effects due to location and year for biomass yield (t ha^{-1}) in 54 Kanlow half−sib (KHS) families across locations (Knoxville and Crossville) and years (2013 and 2014).

Sources	Df	Knoxville	Crossville	Combined
	---------- Variance component----------			
KHS	53	1.67**	2.26**	0.96*
Rep†/Rep [Location]	2 (4)‡	0.04	1.77	0.90
KHS × Location	53	-	-	0.99*
KHS × Year	53	0.44	0.13	0.30
KHS × Rep/Rep [Location]	106 (212)‡	1.43**	2.85**	2.14***
KHS × Year × Location	53	-	-	-
KHS × Year × Rep [Location]	108 (216)‡	1.68***	5.02***	3.28***
Plant [KHS]§	df ¶	28.44***	45.53***	37.24***
	---------- Test of fixed effects (F−values) ----------			
Year	1	379.32***	357.64***	612.52***
Location	1	-	-	87.56**
Location × Year	1	-	-	10.01**

*Significant at $p < 0.05$.
**Significant at $p < 0.01$.
***Significant at $p < 0.0001$.

†Rep, Replication.
‡The df in parentheses indicates the df for combined locations.
¶df (Knoxville = 268, Crossville = 214, Combined = 552).
§Estimated from 2013 individual−plant yield data which was obtained from five plants per plot using five tiller weight; two replications from Knoxville and one from Crossville

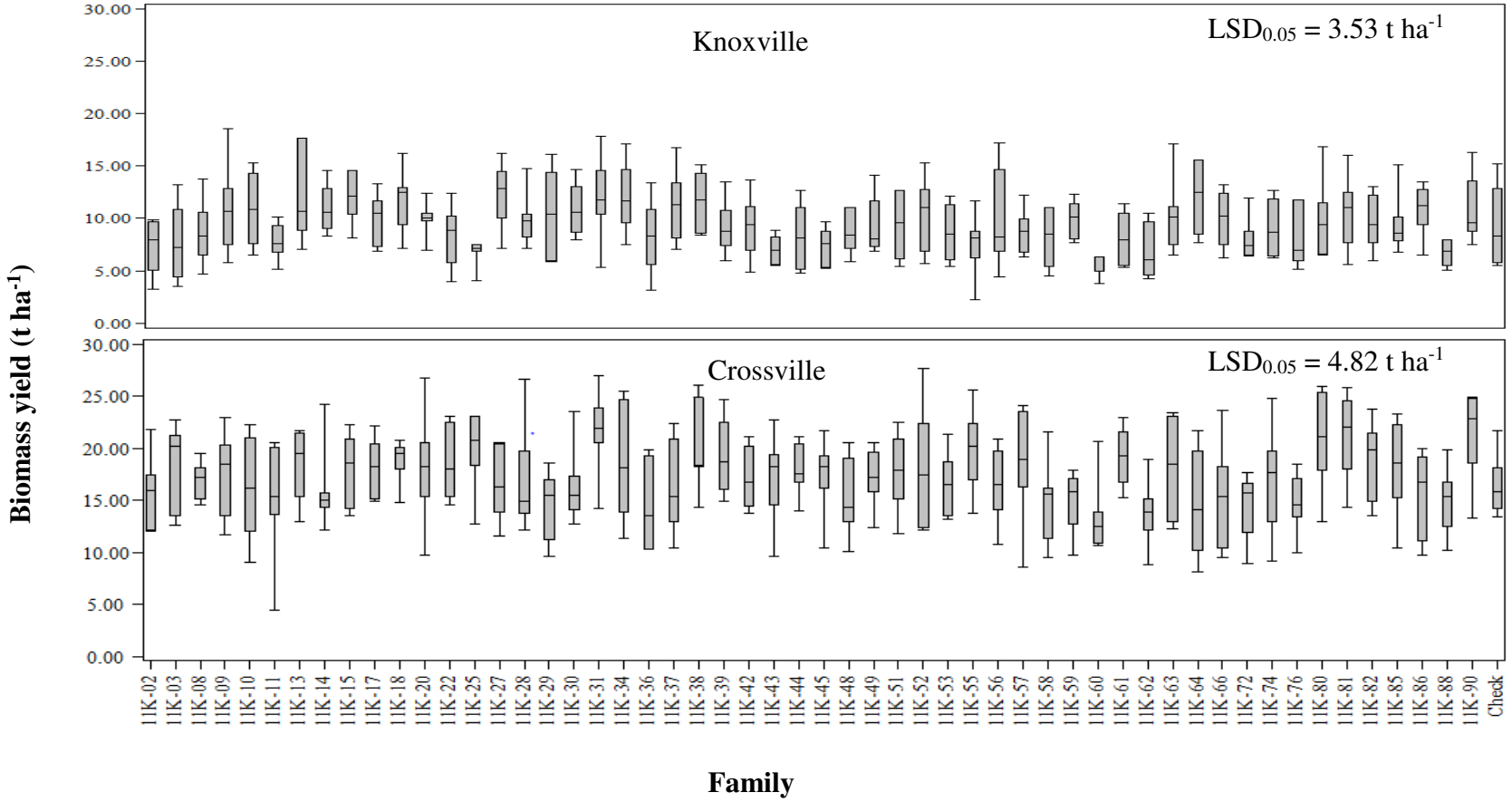


Figure 1–1. Variation in biomass yield of Kanlow half–sib (KHS) families and Kanlow check at Knoxville and Crossville across 2013 and 2014.

Table 1–2. Variance components and tests of fixed effects due to location and year for morphological and feedstock quality traits in 54 Kanlow half−sib (KHS) families across locations (Knoxville and Crossville) and years (2013 and 2014)‡.

Sources	PH	TA	ST	CL	HC	LG
			---------- Variance component----------			
KHS	5.39	0.04	-	0.01	0.14*	0.06**
Rep†/Rep [Location]	26.25	0.88	0.06	0.02	0.01	0.01
KHS × Location	-	0.01	0.01	-	-	-
KHS × Year	33.09	0.01	0.02	0.06	-	-
KHS × Rep/Rep [Location]	86.98*	0.13**	0.05**	-	-	0.01
KHS × Year × Location	11.25	0.01	-	-	0.06	0.01
KHS × Year × Rep [Location]	192.08***	0.33***	0.11***	0.18***	0.61***	0.26***
Plant [KHS]§	485.28***	1.43***	0.48***	0.95***	1.56***	0.29***
			---------- Test of fixed effects (F−values) ----------			
Year	44.07***	0.08	139.08***	225.35***	88.62***	17.88***
Location	1.04	0.35	10.13	186.42**	52.42*	278.91**
Location × Year	192.42***	0.30	215.93***	283.69***	68.50***	235.90***

*Significant at $p < 0.05$, **Significant at $p < 0.01$, ***Significant at $p < 0.0001$.
†Rep, Replication.
‡ PH, Plant height in cm, from base of the plant to the tip of the longest tiller; TA, Tillering ability in the scaled score (<10 tillers =1 to >80 tillers=9); ST, Stem thickness in the scaled score (1=the thinnest to 5= the thickest); CL, Cellulose in the percentage on dry matter basis; HC, Hemi−cellulose in the percentage on dry matter basis; LG, Lignin in the percentage on dry matter basis.
§Estimated from 2013 individual−plant data which was obtained from five plants per plot using five tiller samples; two replications from Knoxville and one from Crossville.

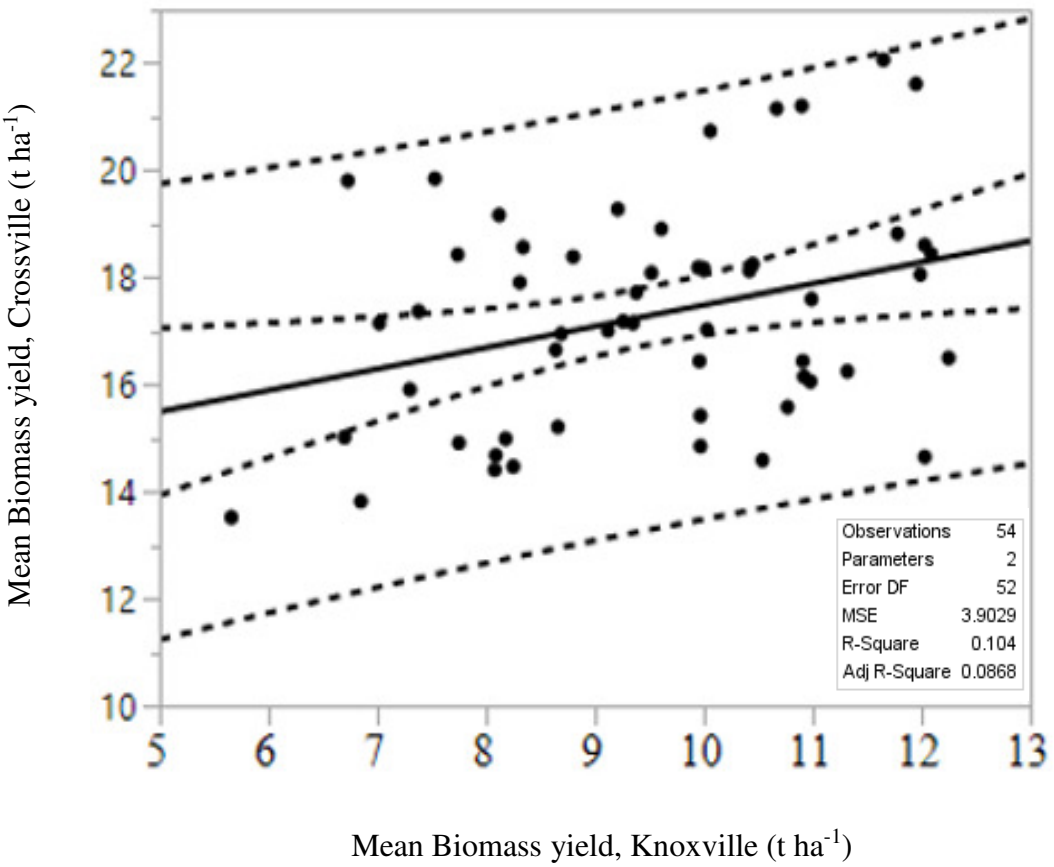


Figure 1–2. Mean biomass yield of 54 Kanlow half–sib (KHS) families demonstrating genotype × environment interaction across years (2013 and 2014). Deviation of dotted plots from the solid line in either direction showed the relative magnitude of genotype × environment interaction effect.

Table 1–3. Summary statistics for measured traits of Kanlow half−sib (KHS) families and Kanlow check across four environments (Knoxville 2013−2014, and Crossville 2013−2014).

Trait†	BMY	PH	ST	TA	CL	HC	LG
	---------- Knoxville ----------						
KHS Mean	9.5	240.2	3.1	3.4	45.5	32.9	6.9
KHS Minimum	2.2	175.8	1.8	1.6	42.3	29.1	5.6
KHS Maximum	18.5	305.0	4.8	5.7	49.2	41.9	8.9
Kanlow Check	9.3	249.7	2.8	3.3	45.4	32.5	6.9
$LSD_{0.05}$	3.5	48.5	0.6	1.0	2.5	1.7	1.2
CV%	34.5	11.9	17.5	22.2	3.6	4.1	11.9
	---------- Crossville ----------						
KHS Mean	17.3	245.6	3.8	4.2	43.5	34.6	5.4
KHS Minimum	4.4	209.2	2.4	2.0	41.6	31.6	4.1
KHS Maximum	30.4	293.6	5.0	6.9	45.9	37.2	6.7
Kanlow Check	16.5	237.9	3.5	4.5	42.9	35.6	5.1
$LSD_{0.05}$	4.8	22.1	1.1	2.0	1.3	2.4	1.1
CV%	25.5	5.6	18.1	26.9	1.9	3.9	12.1
	---------- Combined ----------						
KHS Mean	13.4	242.8	3.4	3.8	44.6	33.7	6.3
KHS Minimum	9.6	226.0	3.0	2.9	43.9	32.6	5.6
KHS Maximum	16.9	262.0	4.2	4.5	45.4	35.1	6.9
Kanlow Check	12.9	243.8	3.2	3.8	44.3	33.9	6.1
$LSD_{0.05}$	4.4	25.6	0.7	1.1	1.8	1.6	1.2
CV%	41.1	9.3	21.2	27.4	3.7	4.6	16.9

† BMY, Biomass yield (t ha^{-1}); PH, Plant height (cm); TA, Tillering ability (1−9 scale, 1=the least, 9=the most tillering plant); ST, Stem thickness (1−5 scale, 1=the thinnest, 5= the thickest); CL, Cellulose (%); HC, Hemi−cellulose (%); LG, Lignin (%)

Table 1–4. Estimates of narrow sense heritability of biomass yield and feedstock quality traits in Kanlow half−sib (KHS) families.

Parameter ¶	Biomass Yield	Hemi−cellulose	Lignin
σ_A^2	3.84	0.56	0.24
σ_P^2	39.49	1.76	0.36
h^2	0.10	0.32	0.66

¶ σ_A^2 = additive genetic variance, σ_P^2 = phenotypic variance, h^2 = narrow sense heritability estimated on individual plant basis.

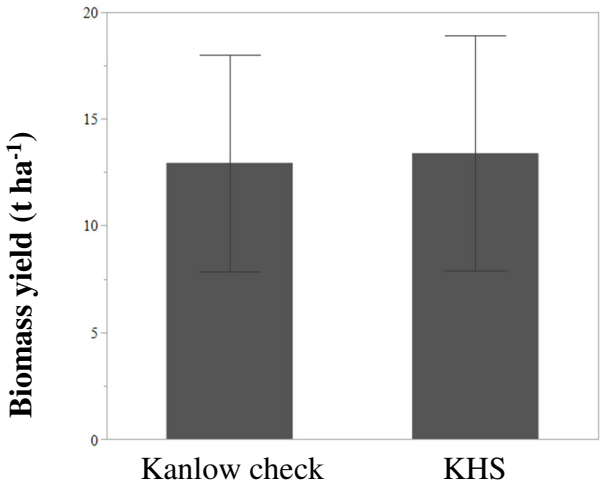


Figure 1–3. Biomass yield of Kanlow half−sib (KHS) families and Kanlow check across two locations (Knoxville and Crossville) and years (2013 and 2014).

Table 1–5. Genetic (r_g) and phenotypic (r_p) correlation and their standard errors among biomass yield, morphological, and feedstock quality traits.

Trait	Biomass yield	Plant height	Stem thickness	Tillering ability	Cellulose	Hemi−cellulose
Plant height (r_g)	0.54 (±0.26)					
(r_p)	0.27 (±0.06)					
Stem thickness (r_g)	0.75 (±0.17)	0.67 (±0.27)				
(r_p)	0.31 (±0.06)	0.29 (±0.05)				
Tillering ability (r_g)	0.07 (±0.26)	-0.77 (±0.33)	-0.56 (±0.27)			
(r_p)	0.19 (±0.05)	-0.04 (±0.06)	-0.01 (±0.06)			
Cellulose (r_g)	0.39 (±0.28)	0.90 (±0.42)	0.98 (±0.33)	-0.19 (±0.37)		
(r_p)	0.16 (±0.05)	0.14 (±0.05)	0.11 (±0.06)	0.07 (±0.05)		
Hemi−cellulose (r_g)	-0.17 (±0.29)	-0.25 (±0.38)	-0.39 (±0.31)	-0.24 (±0.34)	-0.54 (±0.30)	
(r_p)	-0.08 (±0.05)	-0.17 (±0.06)	-0.09 (±0.06)	-0.02 (±0.05)	-0.45 (±0.05)	
Lignin (r_g)	0.44 (±0.20)	0.78 (±0.27)	0.66 (±0.20)	0.01 (±0.26)	0.66 (±0.20)	-0.88 (±0.17)
(r_p)	0.15 (±0.06)	0.17 (±0.06)	0.13 (±0.06)	0.16 (±0.06)	0.60 (±0.04)	-0.51 (±0.04)

Biomass yield (t ha^{-1}); Plant height (cm); Tillering ability (1−9 scale, 1=the least, 9=the most tillering plant); Stem thickness (1−5 scale, 1=the thinnest, 5= the thickest); Cellulose (%); Hemi−cellulose (%); Lignin (%)

Table 1–6. Predicted per−cycle genetic gain (ΔG) for biomass yield, hemi−cellulose, and lignin content.

Selection Intensity	PC§	ΔG¶		
		Biomass Yield (t ha^{-1})	**Hemi−cellulose (%)**	**Lignin (%)**
10%	1	1.10 (8.2)	0.74 (2.2)	0.94 (15.5)
10%	2	2.21 (16.5)	1.49 (4.4)	1.89 (30.9)
15%	1	0.97 (7.3)	0.65 (1.9)	0.83 (13.6)
15%	2	1.95 (14.6)	1.31 (3.9)	1.66 (27.2)

§PC, parental control (1 for the remnant seed of selected half−sibs, 2 for the parental clones of selected half−sib).
¶ $\Delta G = k\ c\ h^2\ \sigma_p$, where, k = the standardized selection differential (for 10%, k = 1.76: for 15%, k=1.55), c = parental control factor, h^2 = narrow sense heritability on an individual plant basis, σ_p = phenotypic standard deviation; parentheses value indicates the gain in percentage.

CHAPTER 2

ASSESSMENT OF GENETIC VARIATION AND IDENTIFICATION OF QTL ASSOCIATED WITH REGROWTH VIGOR IN LOWLAND SWITCHGRASS

Abstract

Switchgrass (*Panicum virgatum* L.) is a warm−season perennial grass species which is recognized for forage and feedstock production in the USA. Stability of biomass yield and regrowth vigor under changing harvest frequency would help to deal with potential fluctuations in the feedstock market and provide a continuous supply of quality forage for livestock. Genetics underlying regrowth vigor is considered to be quantitatively inherited. Quantitative trait loci (QTL) mapping is a powerful method for determining the genetic control of a quantitative trait and expedite genetic improvement. The objectives of this study were (i) to assess genetic variation associated with regrowth vigor, and (ii) to identify QTL associated with regrowth vigor after multiple cuttings in lowland switchgrass. A nested−association mapping (NAM) population comprising 2000 pseudo F_2 progenies were evaluated for regrowth vigor. The result showed significant variation among NAM families for regrowth vigor ($P < 0.05$). A linkage map comprising 2,684 single nucleotide polymorphism (SNP) markers were generated that distributed into 18 linkage groups. The map covered a total length of 3,119 cM with individual chromosome sizes ranging from 124 cM (chromosome 8B) to 252 cM (chromosome 4B) with the marker density of one SNP every 1.3 cM distance. The association between regrowth vigor and genotype was analyzed by composite interval mapping. A total of 10 QTL were detected in 6 chromosomes 1B, 5A, 5B, 6B, 7B, and 8A. The phenotypic variation explained by each QTL ranged from 1.6% to 4.7%. The additive genetic effects of individual QTL ranged from −0.13 to 0.26. No single QTL showed a markedly large effect suggesting complex genetics underlying regrowth vigor in switchgrass. SNP markers closely linked to QTL could be used for marker−assisted breeding. Several candidate genes were identified in the vicinity of QTL that play a variety of roles in developmental processes including plant hormonal signal transduction,

nucleotide biosynthesis, secondary metabolism, senescence, and responses to both biotic and abiotic stresses.

Introduction

Switchgrass (*Panicum virgatum* L.) is a warm−season, perennial grass which is recognized as an important component of the North American tallgrass prairie (Vogel, 2004). The natural population of switchgrass is reported to exist across a broad range of environments. Based on the natural habitat, switchgrass cultivars are mainly classified into two distinct ecotypes, lowland and upland (Porter, 1966; Casler, 2005). Lowland ecotypes are characterized by having taller plant, thicker stems, and are adapted to relatively warm and wet environments compared to upland ecotypes (Vogel, 2004). Switchgrass ecotypes are comprised of varying ploidy levels. Lowland ecotypes are mostly tetraploids (2n = 4x = 36), whereas, upland ecotypes are predominantly octoploid (2n = 8x = 72) (Hopkins et al., 1996).

Switchgrass is identified as a promising grass species for forage and biofuel production because of favorable attributes such as perennial growth habit, ability to adapt in a broad range of environments, and high biomass productivity in marginal land. However, the success of a switchgrass cultivar largely depends on the consistent production of biomass for several years. Several studies have been conducted over the past few decades to understand management practices and the genetic basis that influence biomass production in switchgrass to maximize its utility (Casler, 2012; Nageswara−Rao et al., 2013). Sanderson et al. (2006) suggested three methods to maximize biomass yield, one of which is to optimize harvest timing and frequency. Conventionally, switchgrass is harvested once per year after the killing−frost to achieve maximum yield; however, repeated harvest could increase the total biomass yield by exploiting the regrowth potential of the crop (Monti et al., 2008). Stability of biomass yield and regrowth vigor in switchgrass under changing harvest management would help a switchgrass grower to deal with potential fluctuations in feedstock market and provide a continuous supply of quality

forage for livestock (Sanderson et al., 1999). Switchgrass, like many other native warm−season grasses, can produce high−quality forage. When there is prolonged drought for more than two weeks, all other grasses cease to grow, but switchgrass survives extended period of drought. Because of high forage yield and ability to tolerate extreme drought compared to other native grasses, switchgrass has earned a reputation as an attractive forage crop especially in the mid−south USA (Keyser et al., 2011). Many reports, however, indicate that switchgrass is sensitive to frequent defoliation (Branson, 1953; Neiland and Curtis, 1956; Beaty and Powell, 1976). Frequent defoliation, or clipping, can cause stand reduction and loss of forage yield. Several studies were conducted with an aim to optimize harvest frequency and cutting heights in switchgrass and most of the findings suggest that multiple cuttings especially clipping during fall would significantly reduce long−term survival as well as economic return in lowland switchgrass (Beaty and Powell, 1976; Sanderson et al., 1999; Trócsányi et al., 2009; Kering et al., 2013). Long−term survival of any perennial grass depends on regeneration ability in every growing season. The regeneration ability of perennial grass resides in the dormant crowns and rhizomes which produces growing buds at the time of regrowth (Beaty et al., 1978; Sarath et al., 2014). Therefore, rhizome health plays a very significant role in the long−term survival of perennial grasses. It is understood that multiple cutting significantly reduces the amount of reserves a switchgrass plant could conserve in below−ground biomass which is required for winter survival and transfer of adequate energy to rhizomes for the regrowth in next growing season (Vogel, 2004; Sarath et al., 2014).

The two ecotypes of switchgrass, upland and lowland, have a different response to cutting frequency. A few studies reported an increment of biomass yield under a two−cut system, especially in upland cultivars (Fike et al., 2006; Monti et al., 2008). Upland cultivars have more

cold hardiness, a shorter growth cycle, and higher photosynthetic rates than lowland cultivars (Wullschleger et al., 1996). Greater yield response of upland cultivars with two−cut system is likely associated with such phenological attributes. Because of the shorter growth cycle, the upland cultivars complete the primary period of vegetative growth early in the summer. Therefore, biomass harvest of the upland cultivars early in the summer allowed for the second period of vegetative growth during the fall (Fike et al., 2006). In contrast, the lowland cultivars have a longer vegetative growth period. The lowland cultivars, if clipped in fall, produce many nonrooted shoots which quickly go dormant as soon as temperature drops to resume growth in the following spring (Haferkamp and Copeland, 1984). Nonrooted shoots are vulnerable to be damaged by cold temperature in winter, resulting in reduction of spring growth and overall biomass accumulation in subsequent years. Nevertheless, a few other studies suggested that multiple cutting systems may increase biomass harvest yield in lowland switchgrass depending on cultivar and environmental condition. Mclaughlin et al. (1999) reported that a two−cut system improved biomass yield under favorable weather condition, whereas, the one−cut system was superior in environments where drought was a frequent problem. Robins et al. (2007) reported that the tetraploid alfalfa population exhibit continuous variation in regrowth. Rhizomes, modified underground stems, are carbohydrate storage organs for many perennial plants which determines regrowth vigor in the next growth cycle. Tao et al. (2008) specified that rhizomatousness is controlled by several quantitative trait loci (QTL) having an additive effect. Paterson et al. (1995) reported that regrowth after overwintering is associated with rhizomatousness, a polygenic trait, and identified several QTL associated with this trait in johnsongrass. These studies suggested that regrowth vigor of switchgrass especially under flexible harvest management is possibly a quantitative trait and genotypic variation may also be

influenced by the genotype × environment interaction. Several other molecular studies have identified QTL that controls rhizomatousness in plants such as perennial rice (*Oryza sativa*) and sorghum (*Sorghum bicolor*) (Sarath et al., 2014). Phenotypic selection, for quantitative traits especially those having low heritability, is not efficient as the genotype × environment interaction has a very high confounding effect and often requires laborious phenotyping. To date, little is known on the genetics underlying response to multiple cutting of a switchgrass plant and no study has been exclusively conducted to access genetic variation and identify QTL associated with regrowth vigor especially after increasing the number of cuttings. Identification of genetic markers and application of marker−assisted selection would offer a reliable technique to improve quantitative traits like regrowth vigor leading to improvement in biomass yield under changing harvest frequency.

Therefore, the objectives of this study were,

i.) To assess genetic variation associated with regrowth vigor in lowland switchgrass

ii.) To identify QTL associated with regrowth vigor after multiple harvest in lowland switchgrass

Materials and Methods

Plant Materials

A Nested Association Mapping (NAM) population of switchgrass, developed by the Nobel Research Institute, LLC in Ardmore, Oklahoma, was used in this study. The NAM population was developed by crossing fifteen diverse lowland switchgrass genotypes (Table 2−1) to a common parent 'AP13'. Fifteen diverse genotypes were used as the pollen parent and AP13 as a common maternal parent. AP13 was used as a common maternal parent because it is the genotype whose genome is being sequenced and widely used in switchgrass genomics research.

An additional reason for using a common maternal parent was to maintain a consistent maternal effect across all crosses. To make crosses, AP13 was clonally propagated, and a copy of AP13 was grown alongside each of the diverse parental genotypes in the greenhouse at 32°C/21°C day/night temperature and 16 h photoperiod. Upon flowering, inflorescences from each pair of parents (AP13 and one of the diverse parental genotypes) were bagged together, and seeds were harvested separately. This initial cross generated fifteen 'AP13 × diverse genotype' F_1 families. From each resulting 'AP13 × diverse genotype' F_1 family, 10 F_1 plants were raised and chain crossed with one another to generate recombinant chain−cross families (Figure 2−1). In the chain cross, the inflorescence of the first and the second F_1 plants were bagged together, then second and third F_1 plants were bagged together, and so on with final bagging between the tenth and the first F_1 plants. Thus, 10 recombinant chain−cross families within each of 'AP13 × diverse genotype' F_1 family were generated. Twenty random plants were selected from each chain−cross to generate 200 chain−cross progenies (10 chain−cross × 20 plants = 200 per 'AP13 × diverse genotype' cross). However, eight 'AP13 × diverse genotype' F_1 families out of fifteen did not produce the required number of F_1 seedlings; thus only 5 F_1 plants were chain crossed, and fifteen random plants were selected from each chain−cross to generate 75 chain−cross progenies (5 chain−cross × 15 plants = 75 per 'AP13 × diverse genotype' cross). Therefore, the NAM population comprised of 2000 randomly selected chain−cross progenies (200 chain−cross progenies generated from each of the seven 'AP13 × diverse genotype' F_1 families, and 75 chain−cross progenies from each of the eight 'AP13 × diverse genotype' F_1 families) (Table 2−2. Figure 2−1). The chain−cross progenies hereafter referred to as 'pseudo F_2 progenies' that created the 'NAM Population'.

The 15 diverse genotypes which were initially crossed with AP13 hereafter referred to as 'founder parents'. Similarly, F_1 plants generated from each of the AP13 × diverse genotypes cross and used in the chain cross to generate pseudo F_2 progenies hereafter referred to as 'chain−cross parents'. A total of 2350 plants, including 2000 pseudu F_2 progenies (NAM population), 30 copies of AP13, three copies of each founder parents (3 x 15 = 45), two copies of each chain−cross parents (2 x 135 = 270), and five copies of Alamo check, were planted in the field at two locations Knoxville, TN and Ardmore, OK with two replications at each location for biomass yield evaluation. In this study, the plant materials were evaluated for regrowth vigor in the Plant Science Unit (35°54'1" N 83°57'17" W) of East Tennessee Research and Education Center (ETREC) in Knoxville, TN.

Field Experiment and Phenotypic Data Collection

The NAM population along with the founder parents, chain−cross parents, AP13, and Alamo check were planted at Plant Science Unit of ETREC, Knoxville, TN which is located at an elevation of 259 m. The type of soil is a Shady loam (fine−loamy, mixed, subactive, thermic Typic Hapludults). The field experiment was planted in two replications using an alpha lattice design with the plant to plant spacing at 0.90m. To minimize border effects, border plants were planted all around the main plots. In each replication, the plant materials were accommodated in a block of 47 rows × 50 plants. Because of the insufficient number of ramets produced, only one replication was planted in 2013 while the other replication was established in 2014. Replication 1 was planted in June 2013 while replication 2 was established in July 2014. The field nursery was treated with pre−emergence herbicide, Dual II Magnum (Metolachlor; Syngenta Crop Protection, Inc., Greensboro, NC) at the rate of 2.84 L ha^{-1}, and Prowl H_2O (Perdamethalin; BASF Corporation, Research Triangle Park, NC) at the rate of 3.31 L ha^{-1} during spring of each year

until 2017. Post−emergence herbicide, 2,4−D at the rate of 2.37 L ha^{-1} with surfactant at the rate of 1.18 L ha^{-1}, was applied approximately 60 days post transplanting of field nursery. The field nursery was not supplemented with any fertilizer during the establishment year. During post−establishment years, the field nursery was amended with 60 Kg ha^{-1} N each spring until 2017.

The experiment of the NAM population was originally established for the US Department of Energy (DOE)−funded project and harvested under one cut system during the project period from 2013 through 2016. Afterward, with the objective to understand genetics underlying response of a switchgrass plant after an increased number of cutting, the experiment was clipped twice per year for the current study. In the year of 2017, plants were mowed in July and November. Regrowth vigor was recorded in August 2017 thirty days after clipping. In the year of 2018, plants were mowed in August. Regrowth vigor was recorded on September 2018.

Regrowth vigor was recorded in the scale of 0 to 9 (0=no regrowth, 1=poor regrowth, 9=highly vigorous regrowth) (Figure 2−2) from each replication.

Data of replication 1 represent the regrowth vigor of the population after the fourth and fifth year of planting (or, after 5 clipping and 7 clipping) while data of replication 2 represent regrowth vigor after the third and fourth year of planting (or, after 4 clipping and 6 clipping).

Genotyping and Linkage Map Construction

Genotyping of NAM population was carried out in Dr. Shawn Kaeppler's laboratory at the University of Wisconsin, Madison, WI. A two−step process was involved in genotyping of the NAM population. First, genomic sequence data of NAM founder parents were generated using exome−capture method and the resulting sequences were aligned with the switchgrass reference genome, AP13 (*Panicum virgatum* v1.1, DOE−JGI, http://phytozome.jgi.doe.gov/) to identify SNPs. A total of 25.7 million SNPs was identified in the genomes of NAM founder

parents out of which only 13,451 SNPs were present across all parents covering 18 chromosomes. Initially, segregation of all SNPs was tested using chi−square test. Markers whose segregation significantly departed from the expected ratio were not used for linkage map construction. The genetic linkage map comprising 2,684 SNPs was constructed using JoinMap (v4.1) software. Kosambi mapping function (Kosambi, 1943) was employed to convert recombination distance between markers into centimorgan (cM) map units. The final linkage map comprising 18 linkage groups was provided by Nobel Research Institute LLC, Ardmore, OK.

Phenotypic Data Analysis

Phenotypic data analysis was performed using the MIXED model analysis (PROC MIXED) in SAS 9.4 (SAS Institute, Cary, NC). In the data analysis model, the year was considered fixed, whereas replication, family, and genotypes within the family were considered random. Least square means of individual genotypes across replications were obtained in a separate model considering genotype as a fixed effect and their statistical differences were detected using Fisher's protected LSD ($p < 0.05$).

QTL Analysis

QTL analysis of regrowth vigor was performed for the year 2017 and 2018 data separately. For QTL analysis, mean values of two replications were calculated in each year. QTL analysis was performed using composite interval mapping (CIM). In this study, CIM was performed using WinQTL Cartographer Ver. 2.5 (Wang et al., 2012). The CIM was run in standard model 6 with five markers as a control in a forward regression model. The window size and walking speed were 10 cM and 1 cM, respectively. The logarithm of odds (LOD) threshold values applied to determine the presence of a QTL. The genome−wide threshold values for

significance ($p < 0.05$) were estimated from 1,000 permutation tests of the dataset for each year as implemented by the program WinQTL Cartographer. In this study, LOD score threshold ≥ 3, derived from the permutation test, was used to declare a putative QTL. The relative magnitude of the effect of each significant QTL was estimated as the percentage of phenotypic variation explained (PVE) by the QTL. The physical map of switchgrass genome flanking 50 kb up−and down−stream region of SNPs linked to the QTL was identified using switchgrass v1.1 annotation information, and candidate genes within this region were scanned using NCBI Blast.

Results and Discussion

Phenotypic Variation

Fifteen founder parents, AP13, and Alamo check exhibited different regrowth vigor (Figure 2−3), suggesting that these parental accessions possessed a different level of regrowth ability. The founder parents, EG 1102−2, EG 1104−1, and PI422006 exhibited the most superior regrowth vigor compared to others. EG 1102−2, EG 1104−1, and PI422006 are originated from the southern region of the United States. The founder parent PI442535 was the least vigorous among all parents, which could be due to the reason that it is an exotic cultivar introduced from Belgium. Alamo check showed the most vigorous growth. Common maternal parent AP13, the genotype which has been originally selected for high phosphorus uptake, displayed poor regrowth vigor regardless of the genetic background of Alamo. This warrants a good research question for future study. AP13 did not differ with founder parent PI442535.

The NAM population displayed nearly continuous variation in regrowth vigor when data were analyzed across two years (Figure 2−4), suggesting the polygenic effect associated with regrowth vigor. Comparison of 2017 and 2018 data showed that the mean regrowth vigor score

of the NAM population in 2018 shifted towards the lower score compared to mean regrowth vigor score in 2017 (Table 2−3), suggesting that the regrowth ability of genotypes declined over the year especially under the system of two clipping per year. The previous study pointed that switchgrass can tolerate a single clipping almost anytime with no year to year reduction in plant vigor; however, two or more clipping per year could reduce crown survival and plant vigor (Beaty and Powell, 1976). Year to year decline in regrowth vigor under a multiple harvest system, as observed in this study, is not surprising because selection for superior regrowth ability under a multiple harvest system has not been attempted in the past.

The analysis of variance of combined data across two years (2017 and 2018) revealed that the NAM families differ in their mean regrowth vigor ($p < 0.05$) (Table 2−4). The significant variation in regrowth vigor among the NAM families indicated that additive genes played a notable role. No variation for regrowth vigor was observed among genotypes within family. It was also evident from the result that the regrowth vigor was influenced by family × year interaction ($p < 0.05$), which means the families that had superior regrowth vigor in the initial year would not necessarily hold the highest ranking in subsequent years. Such flip−flopping is not surprising due to the fact that variation in genotypic response of temperature and precipitation between years could affect switchgrass growth. Effect of temperature and precipitation on switchgrass growth has been well documented (Casler et al., 2004; Bhandari et al., 2010). Genotype within family × replication interaction was evident, although the replication effect was not noticeable. Genotype within family × replication interaction could be due to the reason that genotypes in replication I and II received different clipping frequency. As mentioned earlier, regrowth vigor data were recorded after five and seven clippings in replication I as compared to four and six clippings in replication II. The fixed effect of year was highly

significant ($p < 0.01$) which could be associated with the mortality of some plants and subsequent reduction of vigor of surviving plants in the next growing season under two cutting system. Several studies indicate that switchgrass is sensitive to multiple cutting (Branson, 1953; Neiland and Curtis, 1956; Beaty and Powell, 1976) which is not surprising given the fact that the amount of reserves accumulated in the roots would directly affect rhizomes health and regrowth of switchgrass in next growing season (Sarath et al., 2014).

The mean for regrowth vigor and the range across two years are presented in Table 2−3. Regrowth vigor score of NAM population ranged from 0 to 9 with a mean score of 2.5. Overall mean regrowth vigor score of the 15 founder parents was 3.6, and it ranged from 0 to 9. The chain−cross parents had a mean regrowth vigor score of 3.5 with a range from 0 to 9. The common maternal parent, AP13, has a mean regrowth score of 2.5, whereas, Alamo check had a mean score of 5.1. Mean separation of the combined data revealed that regrowth vigor of the NAM population differed with founder parents, chain−cross parents, and Alamo check ($p < 0.05$). Regrowth vigor of the NAM population did not show any difference with AP13. Likewise, no difference was detected between founder parents and chain−cross parents. A similar trend was observed in 2017. This trend was also true in 2018 except that regrowth vigor of founder parents was not different from Alamo check. Both founder parents and chain−cross parents showed superior regrowth vigor than AP13. The results suggest an event of heterosis due to complete dominance as chain−cross parents were originated from AP13 × founder parents cross and showed regrowth vigor similar to founder parents but superior to AP13. However, it was not clear why heterosis exhausted in NAM population so abruptly resulting in a poor regrowth vigor compared to both founder parent and chain−cross parent. One explanation of this is that it may be the case due to sib mating. Further, C−scaling test ($4F_2$-$2F_1$-P_1-P_2=0) reveals that additive−

dominance model of inheritance is not adequate to explain these data which indicates the genetic complexity associated with regrowth vigor possibly due to non−allelic interaction or abnormal chromosome behavior. In the future, in−depth study of heterosis effect and the role of non−additive genes would be beneficial for improving regrowth vigor. Among all, Alamo was the most consistent genotype that showed high regrowth vigor. Alamo switchgrass was originally selected for use in pasture (Kaiser, 2009) which may have resulted in indirect selection of regrowth vigor.

Overall mean regrowth vigor score of the NAM population declined by 31% in 2018 compared to 2017. The trend of falling−off regrowth vigor was also true for founder parents, chain−cross parents, AP13, and Alamo check. Such decline in regrowth vigor could be associated with the poor accumulation of reserves in the root during previous year. Plant biomass was harvested under one−cut system until 2016, thereafter, two−cut system in 2017 and 2018. Because of the two−cut system, plants could not complete full growth cycle in 2017. As discussed earlier, previous year plant stand is important for any perennial grass to accumulate reserves in the root which is required for the regrowth in the next growing season.

Genetic Linkage Map

A high−density genetic linkage map was constructed using 2,684 SNP markers. A total of 18 linkage groups were determined. The linkage groups were named according to the name of the chromosomes to which the SNPs were matched with the physical chromosome position on the switchgrass reference genome (*Panicum virgatum* v1.1, DOE−JGI, http://phytozome.jgi.doe.gov/). The map of 18 linkage group covered a total length of 3,119 cM with individual chromosome sizes ranging from 124 cM (chromosome 8B) to 252 cM (chromosome 4B) (Table 2−5, Figure 2−5). The SNPs were evenly distributed along the

chromosome (Figure 2−5). On average, one SNP was mapped every 1.3 cM. The number of markers on different chromosomes ranged from 69 on chromosome 8A to 273 on chromosome 9B. Our map coverage is comparable to the maps published by Tornqvist et al. (2018) and Ali et al. (2019), but larger than two other published maps (Okada et al., 2010; Serba et al., 2013).

Genomic Regions Associated with Regrowth Vigor

For individual year datasets, a total of ten QTL associated with regrowth vigor were identified using composite interval mapping. The QTL were declared when the marker peak exceeded the permuted LOD threshold level. We identified QTL in chromosome 1B, 5A, 6B, and 7B from 2017 dataset, and, in chromosomes 5A, 5B, 6B, and 8A from 2018 dataset (Table 2−6, Figure 2−6). Each QTL was named consisting of abbreviations for the trait name (RV = regrowth vigor), followed by location and year (kn17 = Knoxville 2017, kn18= Knoxville, 2018), and chromosome name (Chromosome = 1 to 9, sub−genome = A or B, and a serial number when there were two or more QTL on the same chromosome). For example, a QTL detected in chromosome 1B was named as *QRV.kn17.1B,* which is found to be linked with marker c1b_53443735 and explained 4.3% of phenotypic variation for regrowth vigor with an additive effect of −0.15. In chromosome 5A, three QTL were detected and named as *QRV.kn17.5A−1*, *QRV.kn17.5A−2*, *QRV.kn17.5A−3* which accounted for a total of 3%, 4.7%, and 3.1% of phenotypic variation and were tightly linked with marker c5a_61728698, c5a_9165315, and c5a_8191879, respectively. Two other QTL identified from 2017 dataset in chromosome 6B and 7B were named as *QRV.kn17.6B* and *QRV.kn17.7B* which explained 3.1% and 3.2% of phenotypic variation with an additive effect of 0.07 and 0.20, respectively. The nearest marker to *QRV.kn17.6B* and *QRV.kn17.7B* were c6b_20112532, and c7b_17213104, respectively. Similarly, QTL identified from 2018 dataset in chromosome 5A, 5B, 6B, and 8A were named as

QRV.kn18.5A, *QRV.kn18.5B*, *QRV.kn18.6B*, and *QRV.kn18.8A* which explained 3.7%, 3.2%, 2.6%, and 3.8% of phenotypic variation, respectively. The closest flanking marker of the QTL, *QRV.kn18.5A*, *QRV.kn18.5B*, *QRV.kn18.6B*, and *QRV.kn18.8A* were c5a_15120636, c5b_71514101, c6b_3764436, and c8a_14434016, respectively. The additive effect of these four QTL were fairly similar (0.12 to 0.16).

A total of five QTL were detected using combined data across years 2017 and 2018. These QTL were named as *QRV.kn.1B*, *QRV.kn.5A*, *QRV.kn.6B*, *QRV.kn.7B*, and *QRV.kn.8A* which explained phenotypic variation ranging from 1.6% to 3.1% and additive effects ranging from −0.13 to 0.19. All of these five QTL were also detected in either the 2017 or 2018 dataset.

Among all QTL identified in this study, only two QTL on chromosome 5A and 6B were stably detected in individual year data as well as combined data across years with a slight shift in position. QTL on chromosome 1B, 7B, and 8A were detected either in 2017 or 2018; however, it was observed that marker peak just failed to exceed the LOD threshold level in the alternative year. This indicates a strong QTL × year interaction effect on regrowth vigor. Indeed, our results showed that family means were influenced by genotype × year interaction (Table 2−3). No QTL was identified to possess a large effect. There is no straightforward answer for this, but we consider a few possible reasons based on our study. First, the phenotypes we studied are dependent on heterozygous multi−locus genotypes, so that many QTL and the variance they explain could go undetected by our methods. Secondly, the regrowth vigor is the result of complex genetic architecture and influenced by genotype × environment interaction. Environmentally responsive QTL were reported in several studies in the past such as for growth related traits in switchgrass (*Panicum virgatum* L.) (Ali et al., 2019), for biomass yield and plant height in switchgrass (*Panicum virgatum* L.) (Serba et al., 2015), and regrowth in sorghum

[*Sorghum bicolor* (L.) Moench] (Murray et al., 2008). Other studies in different species such as in rice (*Oryza sativa* and *O. longistaminata*) (Tao et al., 2008), sorghum (*Sorghum bicolor* and S. *propinquum*) (Washburn et al., 2013), alfalfa (*Medicago sativa* L) (Robins et al., 2007), and wild relative of maize (*Zea mays* ssp. *parviglumis* and *Z. diploperennis*) (Westerbergh and Doebley, 2004) reported the existence of QTL by environment interactions, multiple minor effects, QTL having small effects distributed over several genomic regions, and both male and female parents contributing favorable alleles associated with regrowth ability. These studies reflect the level of complexity associated with this trait.

Mode of Gene Action of the Identified QTL

The mode of gene action of the identified QTL is presented in Table 2−6. Out of 10 QTL identified in this study, four displayed the *d/a* ratio in the range of -0.5 to 0.5, indicating additive gene action. Three QTL seem to exhibit partial dominance ($0.5 < d/a < 1.25$). Moreover, three QTL exhibited overdominance ($d/a > 1.25$) or underdominance ($d/a <-1.25$) gene action. It was not surprising that 60% of the QTL identified in this study exhibited either partial dominance or overdominance given the fact that a heterozygous population like an F_2 hold dominance in addition to additive effects in contrast to double haploids or recombinant inbred lines which lack the dominant effect in QTL (Broman and Sen, 2019). This result suggests that heterosis breeding has a great scope for improving regrowth vigor in switchgrass. Potential of heterosis breeding in switchgrass for biomass yield was demonstrated in past (Vogel and Mitchell, 2008; Bhandari et al., 2017).

Candidate Gene Search

A list of candidate genes, identified by scanning sequence flanking 50 kb upstream and downstream of the major QTL peak markers that were obtained from the physical map of

switchgrass genome (*Panicum virgatum* v1.1), is presented in Table 2−7. Candidate genes were identified based on E−value and identity similarity. E−value provides information about the likelihood that a given sequence match is purely by chance. The lower the E−value, or the closer it is to zero, the more "significant" the match, meaning that candidate genes may share a similar function. An identity percent is a number that describes how similar the query sequence is to the target sequence. The higher the percent identity is, the more “significant” the match. We found at least one candidate gene within a 50 kb upstream or downstream region from the peak marker of each identified QTL with the E−value cutoff $1e^{-4}$ and sequence similarity above 80%. QTL on chromosome 1B is found to have 90% identity similarity (E−value, 0.00) with Serine/arginine−rich splicing factor RSZ21A (SRSF). Duque (2011) described that SRSF plays a key role in the regulation of gene expression which is important to adapt physiological and environmental stress. Candidate gene Phosphoribosylformylglycinamidine synthase, which is essential for *de novo* purine nucleotide biosynthesis, was found to have 97% identity similarity with the QTL, *QRV.kn17.5A−1* on chromosome 5A (Larson and Idnurm, 2010). Another QTL on chromosome 5A, *QRV.kn17.5A−2*, was found to have 91% identity similarity (E−value $2e^{-58}$) with Bowman−Birk type wound−induced proteinase inhibitor *W1P1* which is related to defense mechanism of plant against a pathogen or physical injury (Rohrmeier et al., 1993). The third QTL on chromosome 5A, *QRV.kn17.5A−3* was found to possess similarity with Probable indole−3−pyruvate monooxygenase YUCCA10 with an identity similarity of 90% (E−value, 0.00). Two other QTL identified on chromosome 6B and 7B from the 2017 dataset, *QRV.kn17.6B,* and *QRV.kn17.7B*, have 91% identity similarity with candidate gene AUGMIN subunit 1 (AUG1) and Protein *ECERIFERUM 1* (CER1), respectively. It has been understood that AUG1 plays a critical role in microtubules organization during cell division, whereas CER1 is linked to

responses to biotic and abiotic stresses (Bourdenx et al., 2011; Ho et al., 2011). Similarly, four QTL found from the 2018 dataset in chromosome 5A, 5B, 6B, and 8A were found to have similarity with Probable indole−3−pyruvate monooxygenase YUCCA10 (identity similarity, 90%, and E−value, 0.00), Scarecrow−like protein 9 (identity similarity, 90%, and E−value, 0.00), F−box protein SKIP28 (identity similarity, 95%, and E−value, $5e^{-132}$), and SUMO−activating enzyme subunit 1A (identity similarity, 83%, and E−value, $9e^{-96}$), respectively. Probable indole−3−pyruvate monooxygenase YUCCA10 is reported to involve in auxin synthesis which affects leaves and flower formation (Cheng et al., 2007). Scarecrow−like protein 9 is known to play an important role in development and to cope with either biotic or abiotic stress in plants (Bielskiene et al., 2009). F−box protein SKIP28 is considered to play a variety of roles in developmental processes including plant hormonal signal transduction, secondary metabolism, senescence, and responses to both biotic and abiotic stresses (Zhang and Roberts, 2019). SUMO−activating enzyme subunit 1A mediates the activation of SUMO (small ubiquitin−related modifier) proteins which coordinate gene expression that is necessary for development, and hormonal and environmental responses of plants (Miura et al., 2007). The candidate genes found in this study holds similarities with closely related species Foxtail millet (*Setaria italica*), Sorghum (*Sorghum bicolor*), and Hall's Panicgrass (*Panicum hallii*) (Okada et al., 2010) which signifies the reliability of similar gene function in switchgrass. The functional effect of these candidate genes could be validated by gene expression analysis and used in cultivar improvement.

In summary, a notable variation in regrowth vigor among the NAM families was observed demonstrating the opportunity to improve this trait by exploiting additive genes. The QTL analysis identified ten important genomic regions controlling regrowth vigor which is a

complex trait involving additive and dominant gene action (Tables 2−6). The majority of QTL identified in this study showed dominant, and over−dominant gene action indicating that these QTL have a heterozygous advantage. However, it should be considered that the population used in this study (pseudo F_2) had less power for estimating additive effects, but more for dominant effects. This study represents a first step in understanding the genetics underlying regrowth vigor in switchgrass after multiple harvests. In the future, studies focused on validating the functional effects of the QTL identified in this study could provide great leads in cultivar improvement for high regrowth vigor in lowland switchgrass through molecular breeding.

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Appendix II

Table 2–1. Description of 15 founder parents used for development of Nested Association Mapping (NAM) population.

Accession	Origin	Description	Desirable Trait
PI414065	Arkansas	BN-14668-65	High biomass yield
PI442535	Belgium	156	Rust tolerant
PI421521-1	Kansas	Grin accession of Kanlow	Early plant vigor
PI421521-2	Kansas	GRIN accession of Kanlow	High biomass yield
PI315725	Mississippi	BN-14669-92	Seed retention
PI315723-1	North Carolina	BN-8358-62	Plant height
PI315723-2	North Carolina	BN-8358-62	Seed retention
PI315723-3	North Carolina	BN-8358-62	Early plant vigor
PI422006	Texas	GRIN accession of Alamo	Plant height
EG 1101-1	Georgia	Improved variety derived from Alamo	Compact panicle
EG 1101-2	Georgia	Improved variety derived from Alamo	Early regrowth
EG 1102-1	Georgia	Improved variety derived from Kanlow	Late heading
EG 1102-2	Georgia	Improved variety derived from Kanlow	Rust tolerant
EG 1104-1	Georgia	Improved variety derived from crossing Alamo and Kanlow	Early regrowth
EG 1104-2	Georgia	Improved variety derived from crossing Alamo and Kanlow	Plant height

Table 2–2. Development of Nested Association Mapping (NAM) population comprising pseudo F_2 progenies.

Pollen Parent	Maternal Parent	Pseudo F_1 Family	Pseudo F_2 Progenies
PI414065	AP13	F_1−1	75
PI442535		F_1−2	200
PI421521-1		F_1−3	75
PI421521-2		F_1−4	200
PI315725		F_1−5	75
PI315723-1		F_1−6	200
PI315723-2		F_1−7	200
PI315723-3		F_1−8	200
PI422006		F_1−9	75
EG 1101-1		F_1−10	75
EG 1101-2		F_1−11	75
EG 1102-1		F_1−12	75
EG 1102-2		F_1−13	200
EG 1104-1		F_1−14	200
EG 1104-2		F_1−15	75

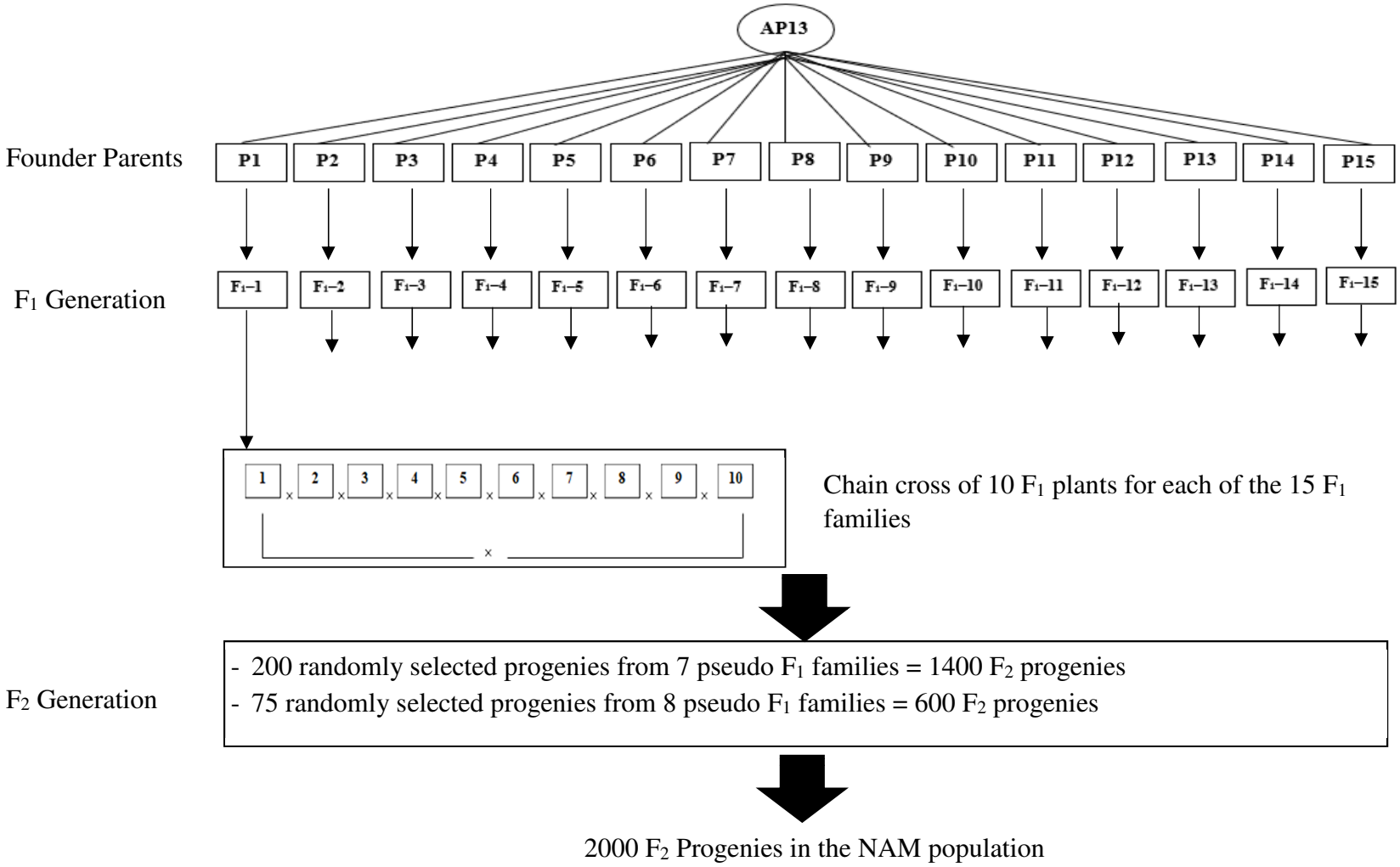


Figure 2–1. Development of Nested Association Mapping (NAM) population.

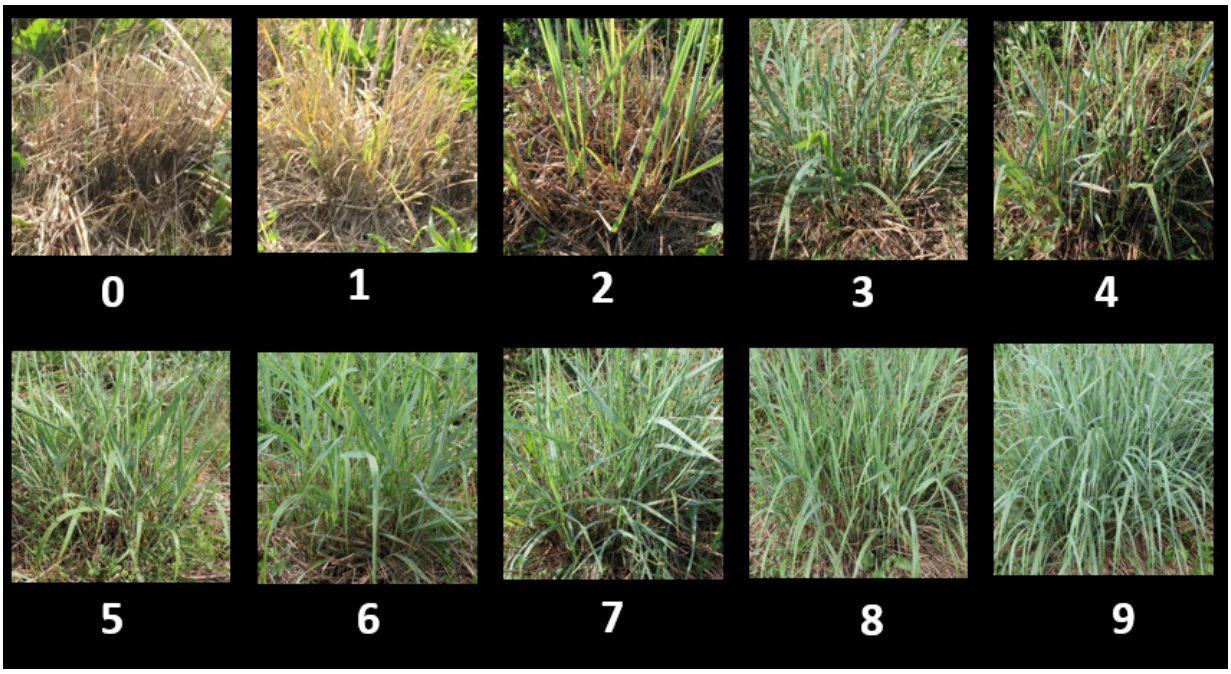


Figure 2–2. Regrowth vigor scoring scale (0 to 9) in switchgrass.

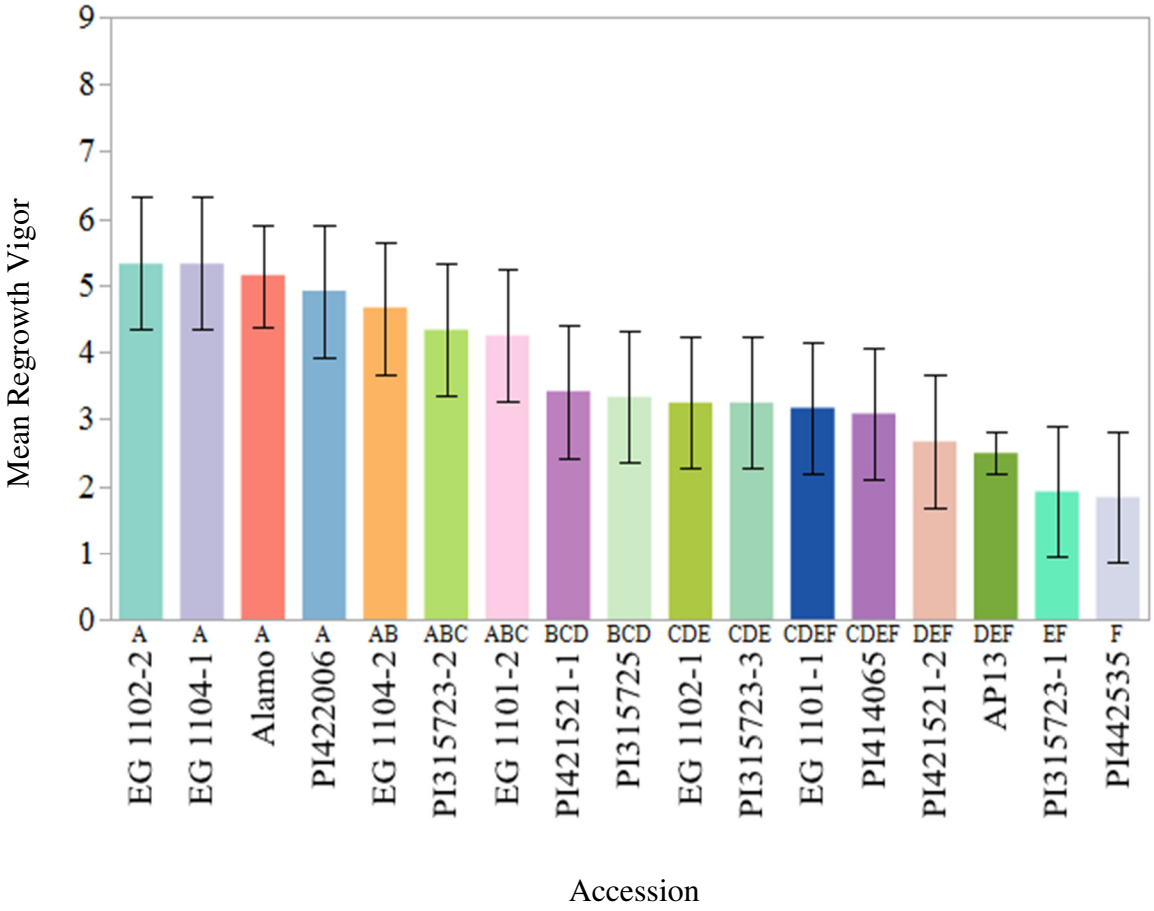


Figure 2–3. Mean regrowth vigor score (0 to 9 scale; 0 = no regrowth, 1= the least vigorous, 9= the most vigorous) and standard error of the founder parents of Nested Association Mapping (NAM) population, AP13, and Alamo check with letter grouping to denote significant difference ($p < 0.05$).

Table 2–3. Summary statistics for measured traits of Nested Association Mapping (NAM) population, Founder Parents, Chain−cross Parents, AP13 and Alamo check.

Population	Regrowth vigor†								
	2017			2018			Combined		
	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
NAM	2.9[a]	0	9	2.0[a]	0	9	2.5[a]	0	9
Founder Parents	4.0[b]	0	9	3.3[bc]	0	9	3.6[b]	0	9
Chain−cross Parents	4.0[b]	0	9	3.0[b]	0	9	3.5[b]	0	9
AP13	2.8[a]	-	-	2.1[a]	-	-	2.5[a]	-	-
Alamo	6.1[c]	-	-	4.2[c]	-	-	5.1[c]	-	-

† Regrowth vigor (score in 0−9 scale; 0=no regrowth, 1=the least vigorous, 9=the most vigorous plant), different letter grouping of mean denotes significant difference ($p < 0.05$).

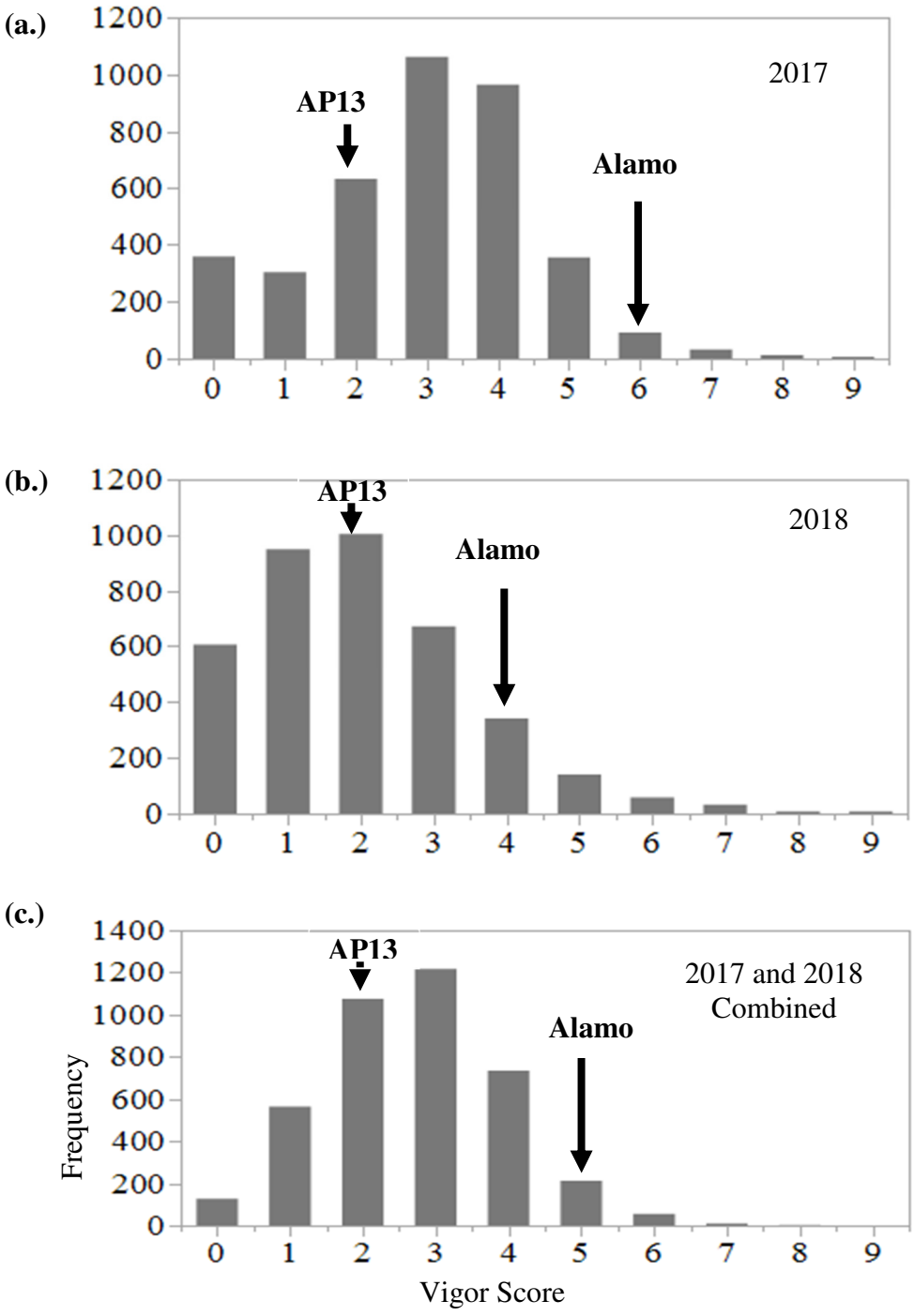


Figure 2–4. Frequency distribution of Nested Association Mapping (NAM) population for regrowth vigor (a.) year 2017 (b.) year 2018, and (c.) across 2 years 2017−2018.

Table 2–4. Variance components and tests of fixed effects due to year of the Nested Association Mapping (NAM) population for regrowth vigor.

Sources of Variation	2017	2018	Combined
	---------- Variance component----------		
Rep†	0.09	0.05	0.05
Family	0.12**	0.09**	0.08*
Genotype (Family)	-	0.53***	-
Family × Year	-	-	0.02*
Genotype (Family) × Rep	-	-	1.82***
Genotype (Family) × Year	-	-	-
Residual	2.52***	2.12***	0.62***
	---------- Test of fixed effects (F−values) ----------		
Year	-	-	302.9***

*Significant at $p < 0.05$.
**Significant at $p < 0.01$.
***Significant at $p < 0.0001$.

†Rep, Replication.

Table 2–5. Single Nucleotide Polymorphism (SNP) markers and genetic distance coverage of 18 linkage group.

Chromosome	SNP Markers	Distance (cM)
1A	167	190.01
1B	199	161.37
2A	217	207.33
2B	171	208.07
3A	120	194.28
3B	122	127.96
4A	99	134.51
4B	130	252.58
5A	183	216.72
5B	146	217.67
6A	121	134.09
6B	91	153.88
7A	152	161.38
7B	90	131.87
8A	69	128.65
8B	97	124.46
9A	237	203.06
9B	273	171.32

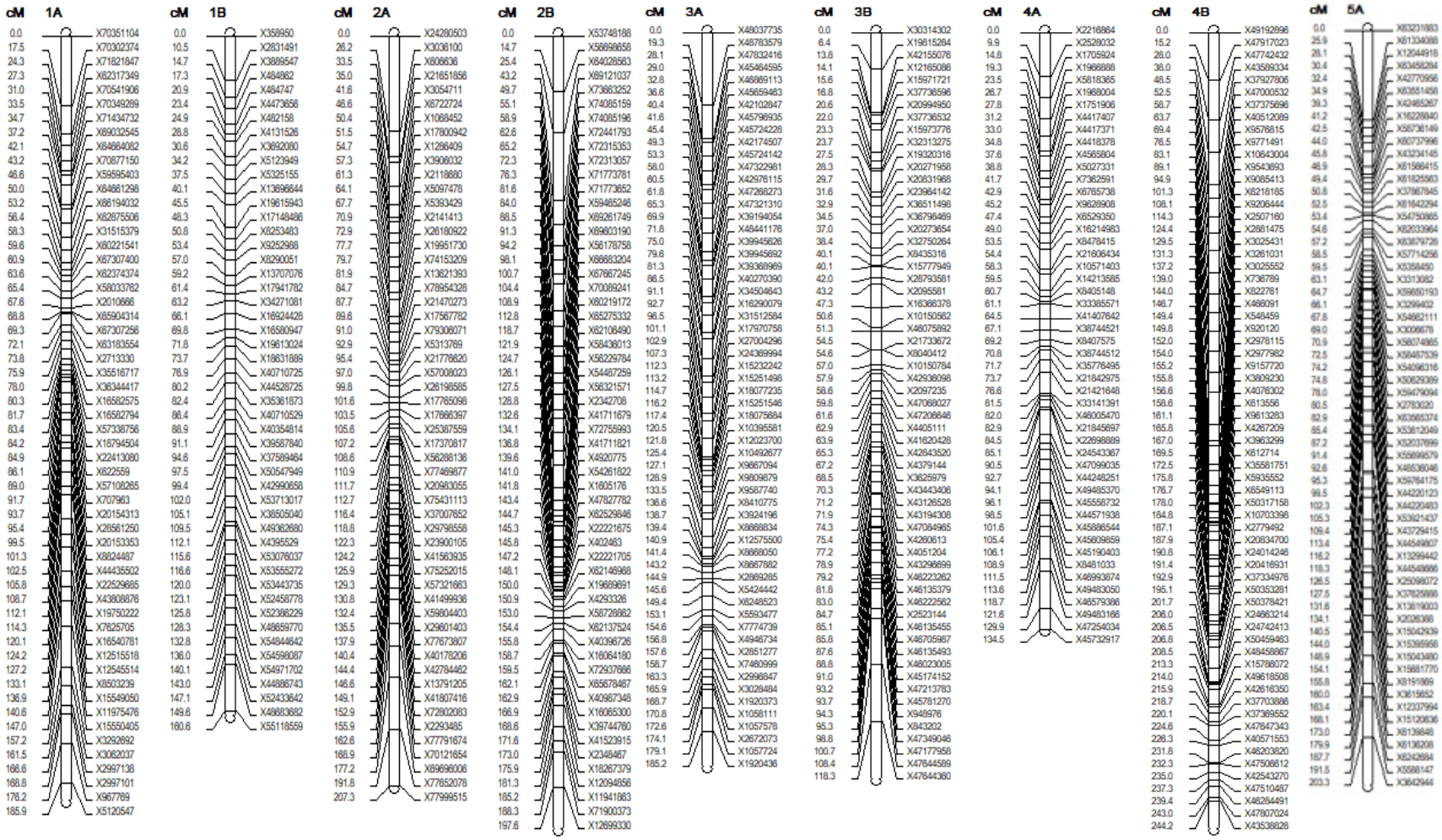


Figure 2–5. Linkage map comprised of 2684 Single Nucleotide Polymorphism (SNP) markers.

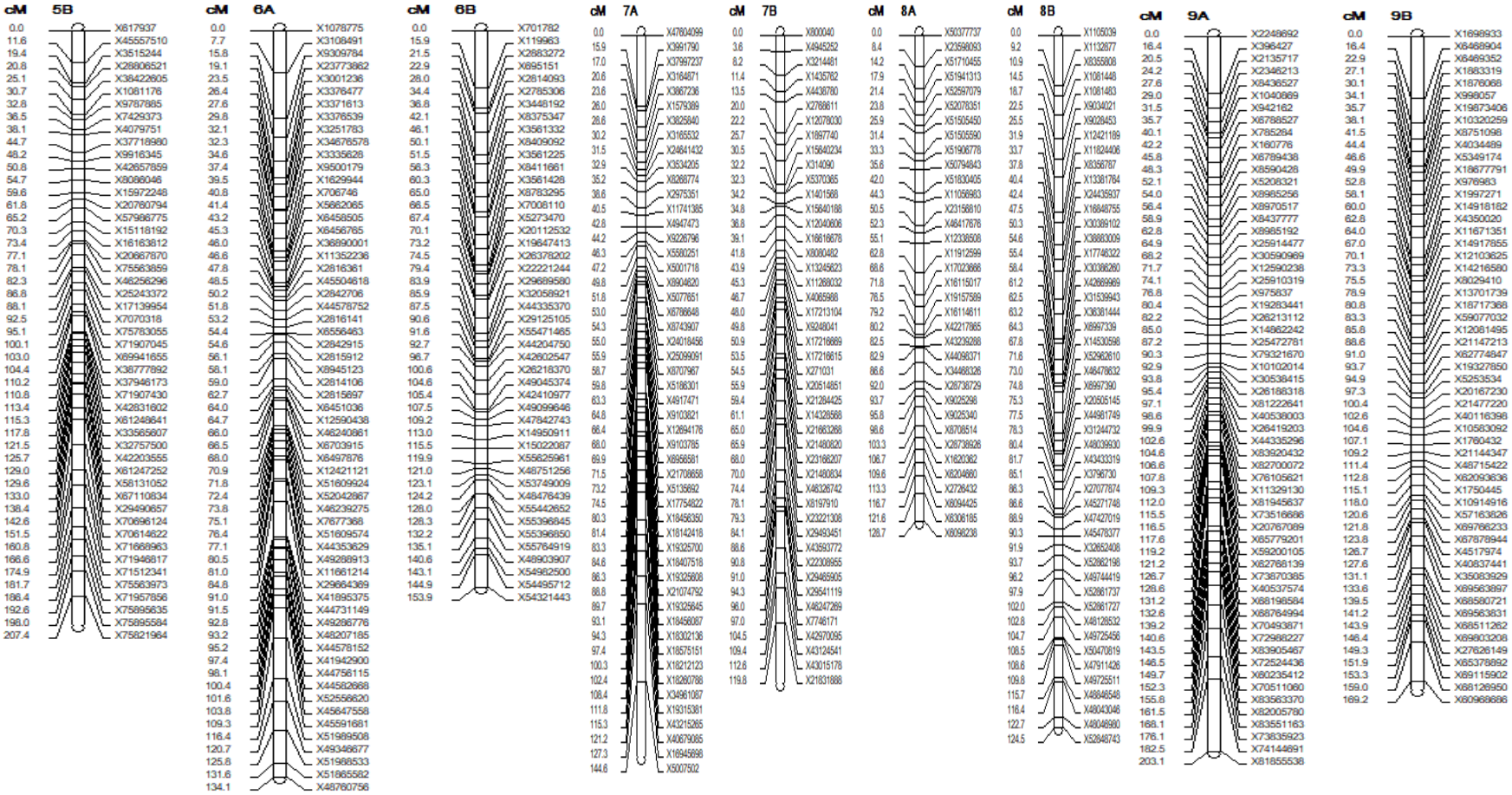


Figure 2–5. (continued).

Table 2–6. Quantitative trait loci (QTL) position, single nucleotide polymorphism (SNP) marker, logarithm of the odds (LOD), additive effect (AE), ratio of the dominance to additive effect (*d/a*), and phenotypic variation explained (PVE) for QTL associated with regrowth vigor identified by Composite Interval Mapping (CIM) in the Nested Association Mapping (NAM) population.

QTL	Chromosome	Environment	Position	Nearest SNP	LOD	AE¶	*d/a*†	PVE (%)
QRV.kn17.1B	1B	2017	120	c1b_53443735	3.6	−0.15	−0.46	4.3
QRV.kn17.5A-1	5A	2017	34.2	c5a_61728698	4.0	0.12	2.08	3.0
QRV.kn17.5A-2	5A	2017	140.6	c5a_9165315	4.1	−0.15	−1.4	4.7
QRV.kn17.5A-3	5A	2017	169.1	c5a_15120636	8.3	0.26	0.38	3.1
QRV.kn17.6B	6B	2017	72.1	c6b_20112532	3.8	0.07	3.57	3.1
QRV.kn17.7B	7B	2017	47.8	c7b_17213104	4.9	0.20	1.00	3.2
QRV.kn18.5A	5A	2018	168.1	c5a_15120636	3.3	0.16	0.37	3.7
QRV.kn18.5B	5B	2018	165	c5b_71514101	3.2	0.12	1.08	3.2
QRV.kn18.6B	6B	2018	48	c6b_3764436	4.5	0.16	1.00	2.6
QRV.kn18.8A	8A	2018	86.7	c8a_14434016	3.1	0.14	0.01	3.8
QRV.kn.1B	1B	Combined	120	c1b_53443735	3.4	−0.13	−0.61	3.1
QRV.kn.5A	5A	Combined	168.1	c5a_15120636	5.5	0.19	0.52	3.0
QRV.kn.6B	6B	Combined	48	c6b_3764436	4.1	0.15	0.86	1.6
QRV.kn.7B	7B	Combined	47.8	c7b_17213104	4.3	0.15	0.98	2.3
QRV.kn.8A	8A	Combined	86.7	c8a_14434016	3.4	0.16	0.14	2.9

¶AE, additive effect; negative AE values indicate that the favorable allele is derived from the maternal parent AP13
†*d/a*, ratio of the dominance to additive effect, d = dominance effect, a = additive effect

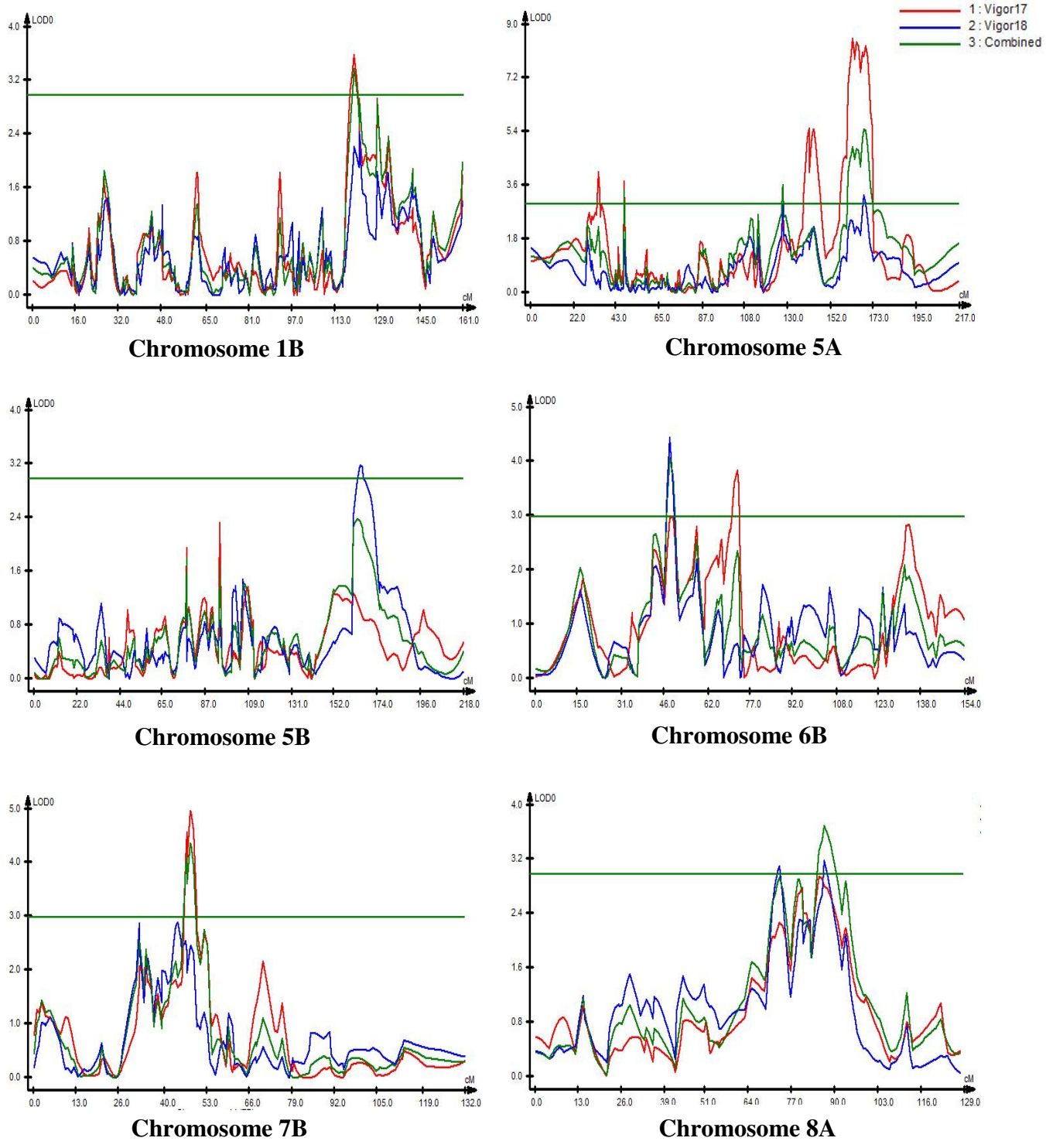


Figure 2–6. Quantitative trait loci (QTL) associated with regrowth vigor identified by Composite Interval Mapping (CIM) using the Nested Association Mapping (NAM) population.

Table 2–7. Candidate genes within range of 50 kb upstream and downstream to the linked marker of the identified QTL associated with regrowth vigor.

QTL	Species	Common name	Description	Identity Similarity	E−value	Accession
QRV.kn17.1B	*Setaria italica*	Foxtail millet	Serine/arginine−rich splicing factor RSZ21A	90%	0.00	XM_004954123.4
QRV.kn17.5A−1	*Panicum hallii*	Hall's panicgrass	Phosphoribosylformylglycinamidine synthase	97%	0.00	XM_025959288.1
QRV.kn17.5A−2	*Sorghum bicolor*	Sorghum	Bowman−Birk type wound−induced proteinase inhibitor W1P1	91%	$2e^{-58}$	XM_002457398.2
QRV.kn17.5A−3	*Setaria italica*	Foxtail millet	Probable indole−3−pyruvate monooxygenase YUCCA10	90%	0.00	XM_004967692.3
QRV.kn17.6B	*Setaria italica*	Foxtail millet	AUGMIN subunit 1	91%	0.00	XM_004972433.2
QRV.kn17.7B	*Sorghum bicolor*	Sorghum	Protein ECERIFERUM 1	91%	$1e^{-141}$	XM_002448115.2
QRV.kn18.5A	*Setaria italica*	Foxtail millet	Probable indole−3−pyruvate monooxygenase YUCCA10	90%	0.00	XM_004967692.3
QRV.kn18.5B	*Setaria italica*	Foxtail millet	Scarecrow−like protein 9	90%	0.00	XM_004970484.3
QRV.kn18.6B	*Setaria italica*	Foxtail millet	F−box protein SKIP28	95%	$5e^{-132}$	XM_004973424.2
QRV.kn18.8A	*Sorghum bicolor*	Sorghum	SUMO−activating enzyme subunit 1A	83%	$9e^{-96}$	XM_002449480.2

CHAPTER 3

IDENTIFICATION OF GENOMIC REGIONS ASSOCIATED WITH SALINITY TOLERANCE IN LOWLAND SWITCHGRASS

Abstract

Switchgrass (*Panicum virgatum* L.) is a promising candidate species for sustainable biofuel feedstock production on marginal land. Soil salinity is one of the significant factors that limit sustainable agricultural production worldwide. The salinity tolerance of plants is a genetically and physiologically complex trait. Salinity can cause a substantial reduction in seed germination, seedling emergence, seedling growth, and yield of switchgrass. This study was designed to investigate genetic variation in lowland switchgrass and identify genomic regions contributing to a high level of salinity tolerance using a subset of a nested association mapping (NAM) population. Salinity tolerance was evaluated based on salt injury score (SIS) using a 1 to 9 scale (1= the most tolerant, 9 = very sensitive) and stress tolerance index (STI) calculated from plant height measurements. Substantial variation among NAM families and genotypes within families was observed ($p < 0.01$) for SIS. The founder parents EG 1104−1, and EG 1104−2 displayed a high level of salinity tolerance which can be used as source material for cultivar development. However, no variation was evident among NAM families and genotypes within families for STI. The association between SIS and single nucleotide polymorphism (SNP) markers was analyzed by composite interval mapping. A total of 5 QTL were detected in 4 chromosomes 2B, 6B, 7B, and 9B. The phenotypic variation explained by an individual QTL ranged from 1.4% to 6.5%. The additive genetic effects of individual QTL ranged from −0.07 to 0.63. Three candidate genes were identified, which were reported to be associated with salinity tolerance. The candidate genes found in this study would provide an excellent foundation to study the functional effects of identified QTL.

Introduction

The US renewable fuel standard (RFS) program, under the energy independence and security act of 2007 (EISA) has mandated a goal to produce 16 billion gallons of cellulosic biofuel by 2022 (www.epa.gov). To contribute the cellulosic biofuel production, several perennials grasses such as switchgrass (*Panicum virgatum* L.), miscanthus (*Miscanthus x giganteus*), napiergrass (*Pennisetum purpureum* Schumach.), reed canarygrass (*Phalaris arundinacea* L.), and bermudagrass (*Cynodon dactylon* L. Pers.) are identified as the potential biofuel feedstocks (Sanderson et al., 2006; Mitchell et al., 2008; Somerville et al., 2010). Among all perennial grasses, switchgrass is recognized as a promising energy crop because of high biomass yield potential, and ability to adapt in a wide range of environments (Vogel, 2004). The natural population of switchgrass can be found from southern Canada through the United States to Mexico. Based on the climatic adaptation, switchgrass is divided into two distinct ecotypes, lowland, and upland (Porter, 1966; Vogel, 2004). Lowland ecotypes are relatively taller and have thicker−stems than upland ecotypes. Lowland ecotypes are adapted to the southern region of North America where the climate is warmer and wetter. Conversely, upland ecotypes are adapted to the northern region where the climate is relatively cold and dry (Porter, 1966; Casler, 2005).

Switchgrass is a promising candidate species for sustainable biofuel feedstock production in marginal land (Vogel, 2004; Zhuo et al., 2015). The marginal lands are characterized by soils having physical and chemical problems such as saline, acid, drought or flood−prone, contaminated soils, highly erodible land, and reclaimed mine−soils (Blanco-Canqui, 2016). Soil salinity is recognized as one of the significant factors that limit sustainable agricultural production worldwide (Yadav et al., 2011; Kim et al., 2012). Salinity may cause low to severe

damage to plant growth and survivability, depending upon plant species and the amount of salt present in the soil. It has been reported that salinity can cause a substantial reduction in seed germination, seedling emergence, seedling growth, and yield of switchgrass (Sun et al., 2018). Therefore, identifying salt−tolerant switchgrass genotypes and improving cultivars having a high level of salt tolerance is very important for maximum utilization of salt−affected lands. Growing bioenergy crops like switchgrass in such marginal land would also help to reduce land competition with growing food crops.

In the USA, over 5 million hectares of irrigated land (Eynard et al., 2005) out of 178 million hectares of arable cropland (Mclaughlin et al., 1999) is estimated to be affected by salinity. Climate change in the global environment has further increased the threat of soil salinization by the incremental rising of sea level (Eynard et al., 2005). The rise of seawater having electrical conductivity over 50 dS m^{-1} may worsen salinity problems in the future. Soil salinity can be categorized in to different classes based on the electrical conductivity (EC) of solute expressed in deci−Siemen $meter^{-1}$ (dS m^{-1}): nonsaline (<2 dS m^{-1}), slightly saline (2–4 dS m^{-1}), moderately saline (4–8 dS m^{-1}), strongly saline (8–16 dS m^{-1}), and very strongly saline (>16 dS m^{-1}) (Rhoades et al., 1999). Typically, EC of soil extract equivalent to 4 dS m^{-1} at 25°C, corresponds to approximately 40 mM concentration of NaCl (Hanin et al., 2016). Different plant species can tolerate a different level of soil salinity (Munns and Tester, 2008). For example, a rice (*Oryza sativa*) plant can be adversely affected when exposed to 50 mM NaCl, whereas, a barley (*Hordeum vulgare*) plant may not necessarily produce any symptom of Na+ toxicity even when exposed to 100 mM NaCl (Negrao et al., 2017). Responses to salinity may even vary within a population of a species because of difference in inherent properties associated with defense mechanisms to minimize salt injury (Gupta and Huang, 2014). Three mechanisms of

salinity tolerance have been known: tolerance to osmotic stress, Na^+ exclusion, and tissue tolerance (Munns and Tester, 2008). Tolerance to osmotic stress refers to the ability of a plant to survive in a high osmotic potential soil environment by maintaining a balance between water uptake by roots and stomatal conductance of leaves for healthy plant growth. The ability of a plant to control Na^+ uptake by its roots to safeguard its accumulation within leaves below a toxic level is referred to as Na^+ exclusion. The ability of plant tissue to compartmentalize the excessive Na^+ at the cellular and intracellular level to avoid toxic concentration within the cytoplasm is referred to as tissue tolerance. Kim et al. (2012) reported that switchgrass could promptly adjust stomatal conductance, osmotic regulation, and compartmentalize excessive Na^+ when exposed to high salt level. It has been demonstrated that switchgrass plants cannot survive when the salt concentration exceeds 400mM in a greenhouse experiment (Kim et al., 2012). Salinity level in the marginal land of the USA is lower than 400mM, but the salt level is building up in arable land every year. It has been estimated that 30% of the land in the USA is vulnerable to a moderate to the severe soil salinity problems by saline seeps (Eynard et al., 2005). Salt−affected areas can be utilized for growing bioenergy crops like switchgrass, where it is not possible to grow food crops. Therefore, it is essential to develop switchgrass cultivars which can tolerate very high levels of salinity.

A range of genetic variation for biomass yield under salinity stress conditions among switchgrass cultivars has been reported (Kim et al., 2012, 2016; Anderson et al., 2015; Sun et al., 2018). Salinity tolerance in plant species is recognized as a quantitative trait (Singh and Gregorio, 2007; Joseph et al., 2010; Lang et al., 2017b). Because salinity tolerance is genetically complex (Koyama et al., 2001), the use of genetic markers would enhance cultivar breeding efficiency (Peleman and Van der Voort; 2003). However, limited research has been done in the

past to understand genetics underlying salinity tolerance in switchgrass and quantify the response of different switchgrass cultivars to the salinity stress. To the best of our knowledge, there is no published study available to date on quantitative trait loci (QTL) mapping for salinity tolerance in switchgrass. Therefore, this study was designed to investigate genetic variation in lowland switchgrass and identify the genomic regions contributing to a high level of salinity tolerance using a nested association mapping (NAM) population. The markers linked to the genomic regions associated with salinity tolerance identified in this study would be useful in marker−assisted selection (MAS) and important resources to expedite breeding for salinity tolerance.

Materials and Methods

Plant Materials

The NAM population of switchgrass developed by the Nobel Research Institute (Ardmore, Oklahoma) was used in this study. The process of NAM population development is presented in Appendix II (Figure 2−1). In brief, fifteen diverse lowland switchgrass accessions were crossed with 'AP13' as a common maternal parent. AP13 was picked as a common maternal parent because it is extensively used genotype in switchgrass genomics research. Using a common maternal parent would also provide a uniform maternal effect across all crosses. Fifteen copies of AP13 were clonally propagated and raised along with fifteen diverse genotypes (Appendix II, Table 2−1) such that fifteen crossing pairs could be formed. All fifteen crossing pairs were grown in the greenhouse at 32°C/21°C day/night temperature and 16 h photoperiod.

Initial crosses were made by bagging inflorescence of each pairs of parents (AP13 and one of the diverse parental genotypes) to generate fifteen F_1 families. Upon maturity, F_1 seeds of 'AP13 × diverse genotype' crosses were harvested, threshed, and stored in separate envelops. For

each ‘AP13 × diverse genotype’ cross, ten F_1 plants were randomly selected, raised in greenhouse, and chain crossed with one another (Appendix II, Figure 2−1). In the chain cross, the first F_1 plant was crossed with the second F_1 plant, then the second F_1 plant was crossed with the third F_1 plant, and so on with the last cross was made between the tenth F_1 plant and the first F_1 plant. The chain cross resulted ten recombinant chain−cross families for each ‘AP13 × diverse genotype’ F_1 family. From each recombinant chain−cross families, twenty plants were randomly selected to generate 200 chain−cross progenies (10 recombinant chain−cross families × 20 plants = 200). This chain cross scheme was true for seven chain−cross families producing 1400 chain−cross progenies. In the remaining eight chain−cross families only 5 F_1 plants were chain crossed because F_1 seedlings were not enough. Chain cross among 5 F_1 plants resulted five recombinant chain−cross families. Subsequently, fifteen plants were randomly selected to generate 75 chain−cross progenies (5 recombinant chain−cross families × 15 plants = 75). The chain cross scheme from eight chain−cross families resulted 600 chain−cross progenies. The chain−cross progenies hereafter referred to as ‘pseudo F_2 progenies’. Therefore, the NAM population comprised of 2000 randomly selected pseudo F_2 progenies (1400 pseudo F_2 progenies from seven ‘AP13 × diverse genotype’ F_1 families, and 600 pseudo F_2 progenies from eight ‘AP13 × diverse genotype’ F_1 families) (Appendix II, Table 2−2. Figure 2−1). The 15 diverse genotypes which were crossed with common maternal parent AP13 hereafter referred to as ‘founder parents’. Similarly, F_1 plants (generated from ‘AP13 × founder parents’ cross) that were chain crossed to generate pseudo F_2 progenies hereafter referred to as ‘chain−cross parent’. The NAM population (2000 pseudo F_2 progenies) was planted in the field along with 30 copies of AP13, three copies of each founder parents (3 x 15 = 45), two copies of each chain−cross parents (2 x 135 = 270), and five copies of Alamo check. The field experiment was established in 2013 at two locations

Knoxville, TN and Ardmore, OK with two replications at each location for biomass yield evaluation.

In this study, a subset of 550 F_2 progeny genotypes originated from a cross between 4 founder parents and AP13 as a maternal parent was used. Population size used in this study is summarized in Table 3−1. Initially, we screened all founder parents and AP13. The results are presented in Figure 3−1. Founder parents 'EG1104−1', 'EG1104−2', and 'PI 421521−1' were found relatively tolerant. Conversely, the founder parent 'PI 315723−1' was the most sensitive. Therefore, we selected a subset of NAM population originating from these four founder parents × AP13 crosses.

Clonal copies of progeny and parental genotypes used in this study were collected from an established field trial at the East Tennessee Research and Education Center (ETREC) (35°54'1" N 83°57'17" W). A clump of each of selected 550 genotypes was collected from the field in fall 2017 and planted in the greenhouse (35°56'38" N 83°56'17" W) for clonal multiplication. All founder parents, including common maternal parent AP13, and Alamo check were also included in the experiment. Plants were planted in cone−tainers (6.9 cm diameter × 17.8 cm depth) (Item code−D27L; Stuewe and Sons, Inc., Tangent, OR) and placed in support trays (Item code−D20T; Stuewe and Sons, Inc., Tangent, OR). The soil media used for planting was Sunshine RSi#1 (Griffin greenhouse supplies, Inc,) and each pot was supplemented with half−teaspoon (~ 5 g) of Osmocot® slow−release fertilizer 14−14−14. The plants were allowed to grow in the greenhouse (30/20 °C day/night temperature; 16h light) for four weeks. Two copies of each genotype were prepared so that one copy could be used for evaluation under salinity stress and other in the control condition. At the end of each experiment, genotypes used in

control block were split and used to produce clonal copies for subsequent experiment. The greenhouse experiment was repeated three times (2017, 2018, and 2019).

Phenotyping for Salinity Stress

The salt treatment was started at E3 to E4 growth stage of plant as described by Moore et al. (1991). E3 and E4 growth stage correspond to the growth stage of the switchgrass plant when 3 and 4 nodes become visible. For the ease of handling, the control set, and salt treatment set were grouped separately in different benches. The genotypes within each set were randomized before the start of the treatment. The set of plant materials used as a control was initially irrigated with 50 ml of normal tap water to maintain the pot moisture at field capacity (20−30% soil moisture). After that, soil moisture was monitored in the individual pots using a moisture meter and irrigated with 5−50 ml of water as needed to maintain soil moisture at field capacity. The plant materials of salt treatment set received 50 ml of 400mM NaCl solution within the first three days. For the first three days, the NaCl solution was added to the pot on a fractional basis to avoid salt stress shock to the plants. We started with 5 ml on the first day and then gradually increased to 15 ml on the second day, and 30 ml on the third day. The pot salinity level was monitored with soil conductivity tester (Soil Test™ Direct Soil Conductivity Tester, Hanna Instruments). After that, 5−50 ml of NaCl solution was added as needed to maintain both soil moisture at field capacity and soil electrical conductivity over 4 dS m^{-1}. The NaCl treatment was continued for 30 days to maintain salinity stress.

Data Collection

Pre−treatment data of plant height was recorded for each plant in both control and treatment set. Data recording for plant height was repeated 30 days post−treatment. Plant height

(cm) was measured from the upper rim of cone−tainer to the tip of the tallest tiller. Plant height data were used to calculate the stress tolerance index (STI).

We estimated the growth of individual plant based on the percentage increase in plant height during the treatment period (30 days). The formula used for calculation is as follows,

$$\text{Percentage increase in plant height} = \frac{\text{Post−treatment height - Pre−treatment height}}{\text{Pre−treatment height}} \times 100$$

STI was calculated using the estimates of percentage increase in plant height of an individual plant under salinity stress relative to the percentage increase in plant height under control condition using formula described by Fernandez (1992) (Ali et al., 2013).

$$\text{STI} = \frac{Y_c \times Y_S}{(Y_{Av})2}$$

Where Y_c = Plant growth in the control condition

Y_s = Plant growth in the stress condition

Y_{AV} = Average growth of population in the control condition

Salt Injury Score (SIS) of individual plants was also recorded in 1 to 9 scales (1=the most tolerant, 9=very sensitive) based on visual appearance that includes green appearance, and vigor of salt−stressed plant 30 days post−treatment.

STI, and SIS data were used for statistical analysis.

Genotyping and Linkage Map Construction

The NAM population was genotyped in Dr. Shawn Kaeppler's laboratory at the University of Wisconsin, Madison, WI. Genotyping of the NAM population was carried out in a

two−step process. In the first step, genomic sequence data of NAM founder parents were obtained using exome−capture method. In the second step, single nucleotide polymorphism (SNP) were identified by aligning these sequence with the switchgrass reference genome, AP13 (*Panicum virgatum* v1.1, DOE−JGI, http://phytozome.jgi.doe.gov/). The sequence alignment revealed that a total of 25.7 million SNPs were distributed in the genomes of NAM founder parents However, only 13,451 SNPs were recorded across 18 chromosomes of all founder parents. Before linkage map construction, marker segregation was tested using a chi−square test. If the markers showed segregation distortion, then they were not included in map construction. JoinMap (v4.1) software was used to create the genetic linkage map which ordered 2,684 SNPs distributed in 18 groups. The recombination distance between markers were then converted into centimorgan (cM) map units by employing Kosambi mapping function (Kosambi, 1943).

Phenotypic Data Analysis

Phenotypic data analysis was performed using the MIXED model analysis (PROC MIXED) in SAS 9.4 (SAS Institute, Cary, NC). In the data analysis model, family and genotypes within the family were considered fixed, and the experiment batch was considered random. Least square means of individual genotypes across three experiments were obtained using the MIXED model analysis in JMP Pro 14 (SAS Institute, Cary, NC) and their statistical differences were distinguished using Fisher's protected LSD ($p < 0.05$). Spearman's correlation coefficient between traits was estimated to observe linear relationship.

QTL Analysis

For QTL analysis, composite interval mapping (CIM) was performed in the computer program WinQTL Cartographer Ver. 2.5 with standard model 6 of Zmapqtl (Wang et al., 2012). As background controls, five markers were used as cofactors in the forward regression method. A

walking speed of 1 cM in the window size of 10 cM was used to scan for significant genomic regions. The threshold for logarithm of odds (LOD) value was determined by 1000 permutation tests (Churchill and Doerge, 1994). QTL analysis of SIS data was performed for the year 2017, 2018, and 2019 data separately. QTL analysis of combined mean data across three years was also performed to compare the individual experiment results. A QTL was considered to be significant at $p < 0.05$ if the peak LOD value exceeded the permuted threshold (LOD 2.5). Additive effects of the detected QTL and the percentage of phenotypic variation explained (PVE) were obtained from CIM results. The transcript sequence flanking 50 kb up−and down−stream regions of tightly linked SNPs was obtained from the physical map of switchgrass reference genome (*Panicum virgatum* v1.1, DOE−JGI, http://phytozome.jgi.doe.gov/) as performed by Ali et al. (2019). The candidate genes within the identified QTL were scanned using NCBI Blast.

Results and Discussion

Phenotypic Data Analysis

A subset of the NAM population was evaluated for salinity tolerance in three greenhouse experiment batches (Figure 3−2). A clonal copy of each genotype was also evaluated under normal condition to calculate stress tolerance index based on growth in plant height. SIS was recorded using a 1 to 9 scale, with 1 indicating the most tolerant and 9 indicating the most sensitive.

Frequency distribution of SIS data was examined for each experiment batch. The distribution of combined SIS data was approximately normal (Figure 3−3). However, STI data did not show normal distribution (Figure 3−3). For QTL analysis, STI data were transformed

using the log10 function to achieve normality of the residuals and hereafter referred to as 'LogSTI'.

The means and range of SIS for NAM, founder parents, AP13, and Alamo check across three greenhouse experiment batches (2017, 2018, and 2019) are presented in Table 3−2. The overall mean SIS of NAM subset was 5.5, and it ranged from 1 to 9. Mean SIS for founder parents PI315723−1, PI421521−1, EG 1104−1, and EG 1104−2 were 8.4, 5.1, 3.0, and 1.8, respectively. The maternal parent AP13 had mean SIS 4.8. Alamo check had mean SIS 4.6. The overall mean SIS of NAM subset was markedly different from mean SIS of founder parents PI315723−1, EG 1104−1, and EG 1104−2 but did not distinguish from maternal parent AP13 or Alamo Check. The founder parents EG 1104−2 showed the highest level of salinity tolerance which was also true in parental screening. The founder parents EG 1104−1 showed the second−best salinity tolerance followed by PI421521−1. The founder parent PI315723−1 exhibited the most sensitive reaction to the salt stress and distantly differed from all other founder parents, AP13 and Alamo check. The poor salinity tolerance of the founder PI315723−1 was also true in each experiment batch as well as parental screening experiment.

The means and range of STI for NAM, founder parents, AP13, and Alamo check across three greenhouse experiment batches (2017, 2018, and 2019) are displayed in Table 3−3. Mean STI of NAM subset was noticeably differed from mean STI of founder parents EG 1104−2, although it did not show any difference with either other founder parents PI315723−1, PI421521−1, and EG 1104−1 nor maternal parent AP13 and Alamo Check. A similar trend was observed in the individual experiment batches. Founder parent EG 1104−2 yielded the best STI value 0.723, which differed from all other founder parents, AP13 and Alamo check. In contrast, founder

parent PI315723−1 possessed the lowest STI value 0.039. No difference was observed for STI value among all other founder parents, AP13, and Alamo check.

Analysis of variance (ANOVA) was conducted to test for variation among families and genotype within the family for SIS and LogSTI. The results are presented in Table 3−4. The NAM families differed in SIS ($p < 0.01$) (Table 3−4). Substantial variation among genotypes within families was also evident ($p < 0.01$) (Table 3−4). The significant variation in SIS among and within the NAM families indicated that salinity tolerance could be improved through recurrent selection. For LogSTI, neither the NAM families nor the genotypes within the family exhibited the notable variation ($p > 0.05$) (Table 3−4).

Relationship Between SIS and STI

STI was calculated based on the relative growth in terms of plant height of the genotypes under salinity condition compared to the control condition. Genotypes having a high value of STI are relatively more salt tolerant than the genotypes having a low value of STI. Regression analysis was conducted to see the relationship between SIS and STI (Figure 3−4). The result suggested that a unit increase in STI would decrease the SIS by an average score of 2.6. Additionally, STI was negatively correlated with SIS (−0.46, $p < 0.01$). STI explained only 17% of the variation for SIS (Figure 3−4).

In this study, plant height was used to calculate STI because shoot growth is considered to be very sensitive in saline condition (Munns and Tester, 2008). However, our result indicated that plant height might not be a good measurement to calculate STI as the majority of SIS variation were unexplained. STI calculation based on yield measurement, was commonly used to evaluate salinity tolerance in several crops (Ali et al., 2013; Kumawat et al., 2017; Jamshidi and Javanmard, 2018; Allel et al., 2019). Some studies in the past reported that the plant height might

increase during the initial period of salt stress as compared to plants grown in control condition (Memon et al., 2010; Qados, 2011). They presented an argument that initial salt stress may induce osmotic adjustment activity and other physiological change in the plant species or the genotype within a plant species to encounter the salt stress resulting in improvement in growth. On the other hand, the negative effect of the salt on the rate of photosynthesis, the change in enzyme activity, and decrease in the level of growth hormone inhibit growth at a later stage (Acosta-Motos et al., 2017). Other study suggested that salt stress caused a continuous decline in plant height (Mathur et al., 2006). Based on such contradictory results regarding plant growth, we speculate that a large portion of variation in plant height among NAM population may not have been captured in our study because height measurement was recorded only once after 30 days of salt treatment. Measuring height at multiple time points could have helped to determine optimal growth stage where STI has more prediction power for the salinity tolerance. In the future, it would be interesting to see if measuring plant height at multiple time points (at least in 10 day intervals) during the salt stress period could provide better precision to interpret the index value.

Genetic Linkage Map

The genetic linkage map is displayed in Appendix II of chapter 2 (Table 2−5, Figure 2−5). A genetic linkage map comprising 2,684 SNP markers was constructed. The SNP markers were arranged into 18 linkage groups. The linkage groups were assigned to chromosomes based on the alignment of SNPs with the physical map available for the switchgrass reference genome (*Panicum virgatum* v1.1, DOE−JGI, http://phytozome.jgi.doe.gov/). A total of 3,119 cM genetic distance was covered by our map with the individual chromosome coverage ranging from 124 cM (chromosome 8B) to 252 cM (chromosome 4B). The map resulted in an average one SNP on

every 1.3 cM distance. In terms of marker distribution, the lowest number of SNPs was mapped in the chromosome 8A (69 SNPs), whereas the highest number in the chromosome 9B (273 SNPs). Several genetic linkage maps were published in past using DNA markers (Okada et al., 2010; Liu et al., 2012; Serba et al., 2013). However, we are able to map larger genetic distance than their maps with a better genome−wide marker coverage. Our map is comparable to the maps published by Tornqvist et al. (2018) and Ali et al. (2019).

Genomic Regions Associated with Salinity Tolerance

Two traits considered in this study were SIS and STI. For the trait, STI, no statistical differences among NAM families or genotypes within a family were observed (Table 3−4). Therefore, QTL analysis was not performed for this trait because the analysis would have no meaning. Using individual experiment batch data, a total of five QTL were identified for SIS by composite interval mapping performed in the WinQTL cartographer. Based on the permutation test, the QTL was declared when the marker peak exceeded the permuted LOD threshold value (LOD 2.5). QTL for SIS were identified in the chromosome 2B, 6B, 7B, and 9B (Table 3−5, Figure 3−5). The identified QTL was designated by using abbreviation for the trait (SIS = salt injury score), followed by location and year (kn17 = Knoxville 2017, kn18= Knoxville 2018, and kn19= Knoxville 2019), and chromosome name (Chromosome = 1 to 9, sub−genome = A or B). Two QTL were detected from the 2017 experiment, and they are located in the chromosome 2B and 9B and named as *QSIS.kn17.2B,* and *QSIS.kn17.9B*, respectively. The QTL *QSIS.kn17.2B* is found to be linked with SNP marker c2b_2342708 and explained 3.7% of phenotypic variation for SIS with an additive effect 0.30. The QTL *QSIS.kn17.9B* is tightly linked with SNP marker c9b_6433764 and explained 3.9% of phenotypic variation for SIS with a negative additive effect 0.07. From the 2018 experiment, one QTL was detected on chromosome 6B was named as

QSIS.kn18.6B which is found to be linked with marker c6b_30777131 and explained 4.6% of phenotypic variation for SIS with an additive effect of 0.48. From the 2019 experiment, two QTL were detected in the chromosome 2B and 7B, which were named as *QSIS.kn19.2B*, and *QSIS.kn19.7B*, respectively. The nearest marker to *QSIS.kn19.2B* and *QSIS.kn19.7B* were c2b_53748188, and c7b_15640188, respectively. The QTL *QSIS.kn19.2B* and *QSIS.kn19.7B* accounted for a total of 1.4% and 6.5% of phenotypic variation with additive effects of 0.30 and 0.63, respectively. The analysis of combined data across three experiment batches (2017, 2018, and 2019) detected only two QTL on chromosome 6A (*QSIS.kn.6A*) and 9B (*QSIS.kn.9B*) which accounted for a total of 5.3% and 5.1% of phenotypic variation with an additive effect 0.40 and −0.20, respectively. The nearest marker to *QSIS.kn.6A* and *QSIS.kn.9B* were c6a_3376539, and c9b_7191347, respectively. The QTL, *QSIS.kn.9B* identified on chromosome 9B using combined data, was detected within close proximity of another QTL detected in 2017 experiment batch which indicates the importance of this genomic region.

It is postulated that the QTL detected in both individual environments and across environments are consistent QTL that may be expressed irrespective of any environmental effect (Serba et al., 2015). However, in this study none of the QTL was stable across three experiment batches or accounted for a substantial effect on phenotypic variation, indicating that they were sensitive to the environments. Salinity tolerance in plants is genetically complex (Koyama et al., 2001). Several studies were conducted in the past to identify QTL in different species such as rice (*Oryza sativa* L.) (Koyama et al., 2001; Lin et al., 2004; Lang et al., 2017a), wheat (*Triticum aestivum* L.) (Asif et al., 2018), maize (*Zea mays* L.) (Luo et al., 2019), and soybean (*Glycine max* L. Merr.) (Lopez et al., 2018). These studies indicated that salinity tolerance in plants is controlled by either a few major genes/QTL or multiple genes with small effects. Koyama et al.

(2001) suggested that a QTL with a small effect may have an enormous effect in a regulatory pathway. Therefore, it is essential to understand the functional effect of the identified QTL to utilize in switchgrass breeding. QTL analysis for traits related to salinity tolerance in switchgrass have not been conducted yet to best of our knowledge. Consequently, QTL identified in this study would provide valuable information for the functional analysis of salinity tolerance in switchgrass. Further, the markers identified in this study may also be valuable resources for MAS in switchgrass breeding program to develop cultivars having a high level of salinity tolerance.

Mode of Gene Action of the Identified QTL

The mode of gene action was estimated by calculating the ratio of dominant to additive effects (*d/a* ratio) (Wegrzyn et al., 2010; Sun and Mumm, 2016). The *d/a* ratio of the identified QTL is presented in Table 3−5. In this study, one out five QTL identified from individual experiment data analysis for the trait SIS displayed partial dominance ($0.5 < d/a < 1.25$), three QTL showed overdominance ($d/a > 1.25$) and remaining one QTL showed underdominance ($d/a <-1.25$) gene action. For combined data analysis, the *d/a* ratio of one QTL displayed additive gene action while other showed overdominance. Most of QTL in this study showed very small additive effects and strong dominance effect as *d/a* ratio either higher than 0.5 or lower than−0.5. The high value of *d/a* ratio indicates that these QTL have a heterozygous advantage, and high levels of dominance can be related to heterosis.

Candidate Gene Search

To search for candidate genes, the transcript sequence flanking 50 kb upstream and downstream of the major QTL peak markers was extracted from the physical map of the switchgrass genome (*Panicum virgatum* v1.1) (http://phytozome.jgi.doe.gov/) and subjected to

Blast in NCBI database under an Expect value (E−value) threshold of $<1e^{-4}$ and sequence identity similarity more than 80%. The list of candidate genes is presented in Table 3−6. We found five characterized and two uncharacterized candidate gene within 50 kb upstream or downstream from the peak marker. These candidate genes are related to closely related species foxtail millet (*Setaria italica*), sorghum (*Sorghum bicolor*), and maize (*Zea mays*). Both uncharacterized candidate genes were located on chromosome 2B. QTL on chromosome 6B is found to have 85% identity similarity (E−value, $1e^{-122}$) with Serine/arginine−rich splicing factor SR45a (SR45A). Serine/arginine-rich proteins are important RNA−binding proteins that play a crucial role in alternative splicing, and regulate pre-mRNA splicing under stress conditions (Butt et al., 2019). SR45A is one of the plant−specific SR−like proteins. Butt et al. (2019) suggested that loss−of−function mutants of SR45 *Arabidopsis* are hypersensitive to salt stress. The Candidate gene SH3 domain-containing protein 2 (SH3P2) was found to have 95% identity similarity (E−value $2e^{-83}$) with the QTL, *QSIS.kn19.7B* on chromosome 7B. Zhuang et al. (2013) reported that SH3P2 functions as an essential regulator of autophagy in *Arabidopsis*. The report further elaborated that autophagy is a catabolic mechanism whereby cytoplasmic materials are engulfed into a structure termed the autophagosome, which plays a vital role in protecting cells against pathogen infection or other unfavorable conditions. Another QTL on chromosome 9B, *QSIS.kn17.9B*, was found to have 93% identity similarity (E−value $1e^{-166}$) with Polyamine oxidase (PAO). Sagor et al. (2016) reported that *Arabidopsis* plant silenced for cytoplasmic PAOs exhibited an increased level of salinity tolerance by reducing reactive oxygen species (ROS) production and actively inducing subsets of stress−responsive genes. The QTL on chromosome 6A, which was detected using combined data, had 90% identity similarity (E−value $2e^{-100}$) with non−specific lipid−transfer protein C4. Pan et al. (2016) demonstrated that

overexpression of lipid−transfer protein improved salt tolerance in foxtail millet (*Setaria italica*). Another QTL, *QSIS.kn.9B*, identified using combined data showed 86% identity similarity (E−value $9e^{-186}$) with Dehydrodolichyl diphosphate synthase (DHDDS) complex subunit NUS1. It is documented that DHDDS is a cis-prenyltransferase which is involved in the biosynthesis of isoprenoids that is necessary for plant growth and survival (Cunillera et al., 2000; Liu et al., 2011). The candidate genes identified in this study were reported to be directly associated with salinity tolerance. Therefore, the findings of this study indicate that salinity tolerance in switchgrass could be improved by manipulating the identified genomic regions through molecular breeding. This study is a step forward in understanding the functional effect of identified QTL.

In summary, the subset of NAM families and genotypes within family exhibited substantial variation for SIS. The QTL analysis revealed five QTL associated with SIS. No single QTL having a large effect was detected for SIS. Nevertheless, the markers linked to QTL identified in this study would be useful for gene pyramiding, and marker−assisted selection to improve salinity tolerance in switchgrass after functional validation of the candidate genes. Functional testing of the genes could be done by overexpressing or down-regulating the target gene through genetic engineering or RNAi or by genetic complementation of a known mutant. The salt−tolerant genotypes identified in this study may be used as a parent material for breeding salt−tolerant cultivars. This study examined only short term (30 days) response for salinity stress. In the future, the entire growth cycle should be observed to acquire more precise knowledge about salinity tolerance in switchgrass.

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Appendix III

Table 3–1. A subset of Nested Association Mapping (NAM) population used for salinity tolerance evaluation.

Male Parent Accession	Origin	Description	Maternal Parent	No. of F_2 Progeny Genotype
PI421521−1	Kansas	Grin accession of Kanlow	AP13	75
PI315723−1	North Carolina	BN−8358−62	AP13	200
EG 1104−1	Georgia	Improved variety derived from crossing Alamo and Kanlow	AP13	200
EG 1104−2	Georgia	Improved variety derived from crossing Alamo and Kanlow	AP13	75
			Total	**550**

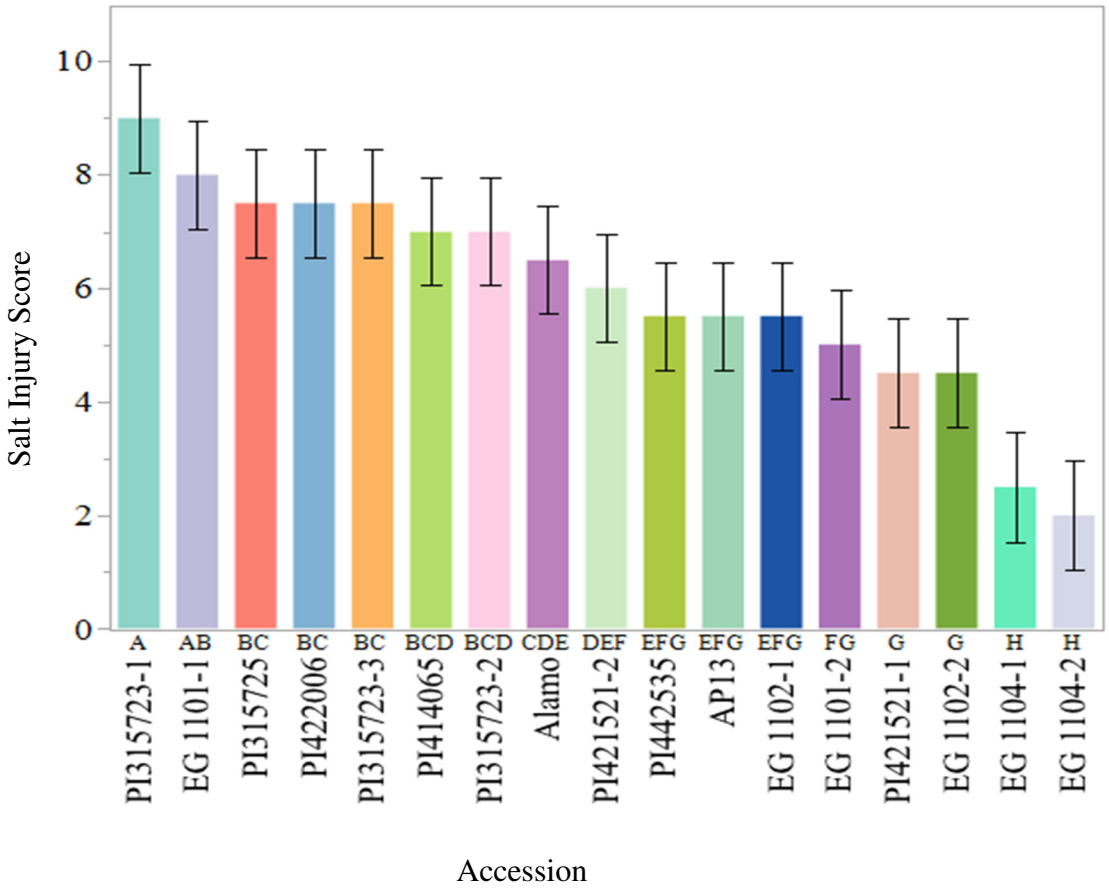
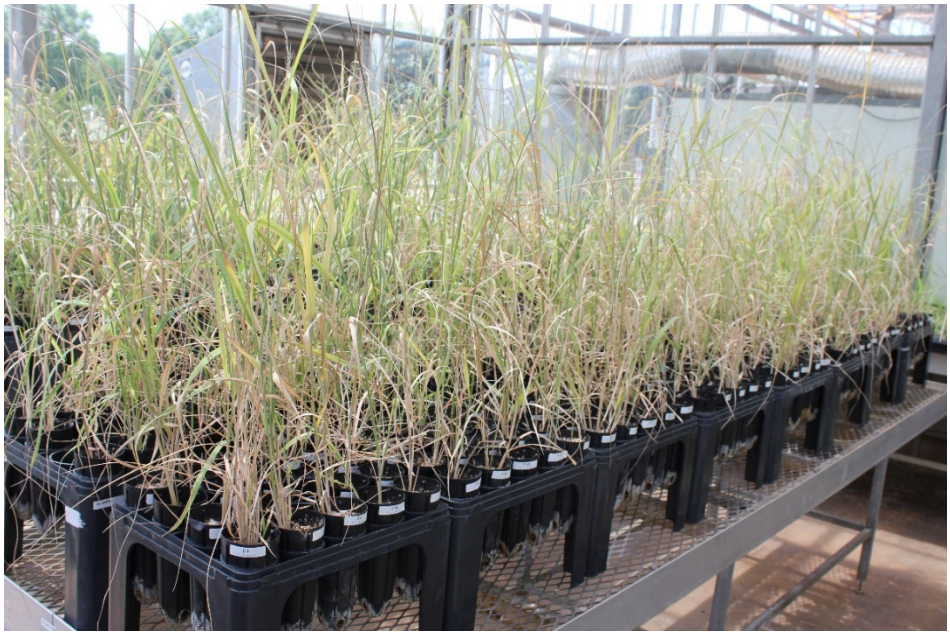


Figure 3–1. Mean Salt Injury Score (1 to 9 scale; 1=Tolerant, 9=Sensitive) and standard error of founder parents of Nested Association Mapping (NAM) population at 400mM NaCl treatment level with letter grouping to denote significant difference ($p < 0.05$).

(a.)



(b.)



Figure 3–2. Greenhouse experiment for salinity tolerance in Nested Association Mapping (NAM) population (a) grown under 400mM NaCl treatment for 30 days (b) grown under control condition.

Table 3–2. Summary statistics of salt injury score (SIS)† of Nested Association Mapping (NAM) population, founder parents of NAM population, AP13, and Alamo Check.

	2017			2018			2019			Combined		
Accession/Population	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**
NAM	4.8^{a}	1	9	5.7^{a}	1		6^{ab}	1	9	5.5^{a}	1	9
PI315723-1	8.7^{b}	-	-	8.3^{b}	-	-	8.3^{a}	-	-	8.4^{b}	-	-
PI421521-1	5^{ac}	-	-	5.3^{a}	-	-	5^{bc}	-	-	5.1^{a}	-	-
EG 1104-1	2.3^{ac}	-	-	4^{ac}	-	-	2.7^{cd}	-	-	3.0^{cd}	-	-
EG 1104-2	1.6^{c}	-	-	2^{c}	-	-	1.7^{d}	-	-	1.8^{d}	-	-
AP13	5^{abc}	-	-	5^{ac}	-	-	4.5^{bcd}	-	-	4.8^{ac}	-	-
Alamo Check	4^{ac}	-	-	4.5^{ac}	-	-	5.5^{abc}	-	-	4.6^{ac}	-	-

†SIS, Salt injury score (score in 1−9 scale; 1=the most tolerant, 9=the most sensitive).
Different letter grouping of mean denotes significant difference ($p < 0.05$).

Table 3–3. Summary statistics of stress tolerance index (STI)† of Nested Association Mapping (NAM) population, founder parents of NAM population, AP13, and Alamo Check.

	2017			2018			2019			Combined		
Accession/Population	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**
NAM	0.314[a]	0	2.855	0.141[a]	0	1.839	0.215[a]	0	2.508	0.224[ab]	0	2.855
PI315723-1	0.008[a]	-	-	0.064[a]	-	-	0.044[a]	-	-	0.039[a]	-	-
PI421521-1	0.154[ab]	-	-	0.233[ab]	-	-	0.218[ab]	-	-	0.202[ab]	-	-
EG 1104-1	0.102[a]	-	-	0.155[ab]	-	-	0.574[bc]	-	-	0.277[ab]	-	-
EG 1104-2	0.819[b]	-	-	0.581[c]	-	-	0.770[c]	-	-	0.723[c]	-	-
AP13	0.238[ab]	-	-	0.220[ab]	-	-	0.254[ab]	-	-	0.238[ab]	-	-
Alamo Check	0.391[ab]	-	-	0.397[bc]	-	-	0.547[abc]	-	-	0.445[bc]	-	-

†STI, Stress tolerance index (higher STI value indicate relatively more tolerant)
Different letter grouping of mean value denotes significant difference ($p < 0.05$).

Table 3–4. Sources of variance on salt injury score (SIS) and Log10 transformed data of salinity tolerance index (LogSTI) for the Nested Association Mapping (NAM) population across three greenhouse experiments (2017, 2018, and 2019).

Sources of Variation	df	Type III MS	F−value	Significance
SIS				
Family	3	18.5	5.22	**
Genotype (Family)	540	5.3	1.48	***
Batch†	2	224.6	63.48	***
LogSTI				
Family	3	0.24	0.85	*ns*
Genotype (Family)	538	0.31	1.10	*ns*
Batch	2	7.55	26.47	***

ns = not significant
*Significant at $p < 0.05$.
**Significant at $p < 0.01$.
***Significant at $p < 0.0001$.
† Batch denotes the greenhouse experiment conducted in 2017, 2018, and 2019.

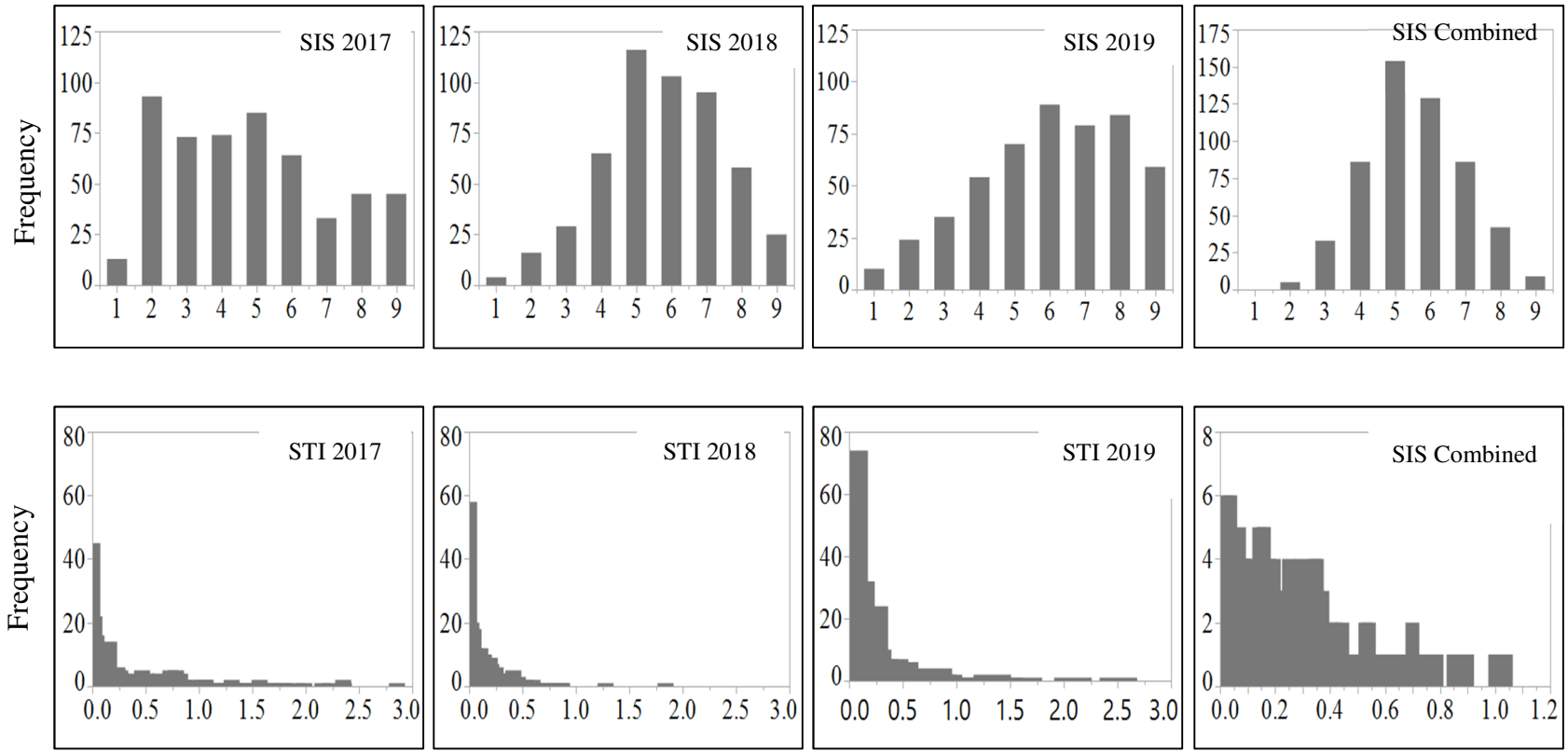
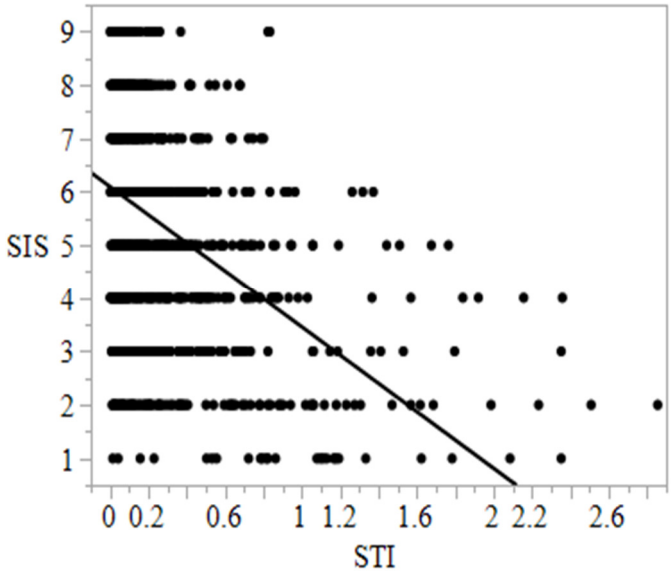


Figure 3–3. Frequency distribution of salt injury score (SIS), and salinity tolerance index (STI) of Nested Association Mapping (NAM) population.



SIS = 6.096 – 2.641 × STI

Rsquare = 0.17

Spearman's correlation =–0.46***

Figure 3–4. Relationship between mean salt injury score (SIS), and salinity tolerance index (STI) across three experiments (2017, 2018, and 2019).

***Significant at $p < 0.0001$.

Table 3–5. Quantitative trait loci (QTL) position, single nucleotide polymorphism (SNP) marker, logarithm of the odds (LOD), additive effect (AE), ratio of the dominance to additive effect (*d/a*), and phenotypic variation explained (PVE) for QTL associated with salinity tolerance assessed by salt injury score[§] (SIS) in the Nested Association Mapping (NAM) population.

QTL	Chromosome	Environment	Position	Nearest SNP	LOD	AE¶	*d/a*†	PVE (%)
QSIS.kn17.2B	2B	GH 2017	128.7	c2b_2342708	2.9	0.30	−2.6	3.7
QSIS.kn17.9B	9B	GH 2017	47.6	c9b_6433764	2.9	−0.07	3.71	3.9
QSIS.kn18.6B	6B	GH 2018	90.4	c6b_30777131	3.7	0.48	1.66	4.6
QSIS.kn19.2B	2B	GH 2019	0.01	c2b_53748188	2.6	0.30	2.7	1.4
QSIS.kn19.7B	7B	GH 2019	34.8	c7b_15640188	4.2	0.63	1.01	6.5
QSIS.kn.6A	6A	Combined	30.9	c6a_3376539	3.3	0.40	−0.4	5.3
QSIS.kn.9B	9B	Combined	58.5	c9b_7191347	3.6	−0.20	1.7	5.1

§ Salt injury score (SIS), measured in 1 to 9 scale, 1 = tolerant, and 9 = very sensitive.
¶AE, additive effect; negative AE values indicate that the favorable allele is derived from the maternal parent AP13.
† *d/a*, ratio of the dominance to additive effect, d = dominance effect, a = additive effect.

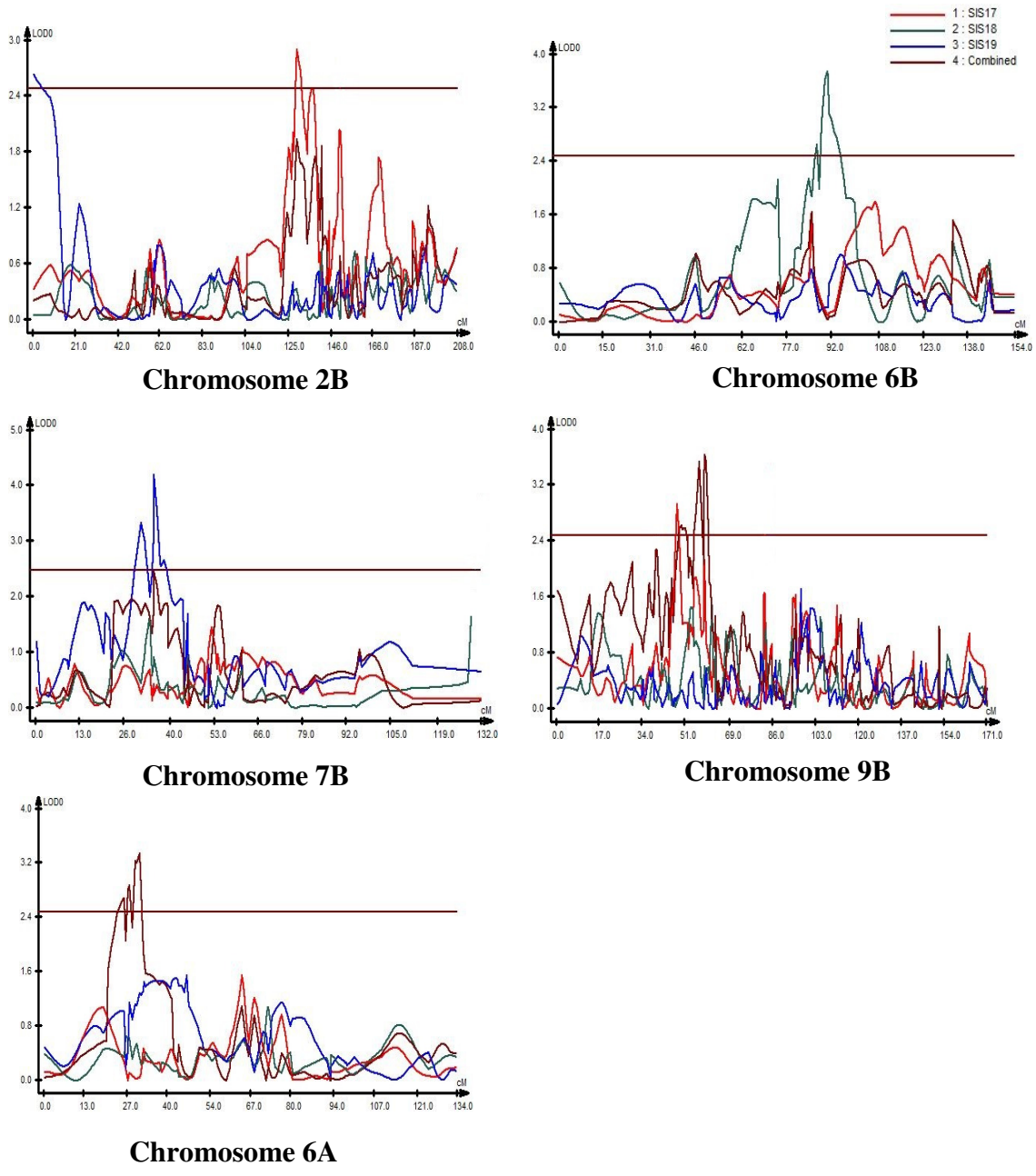


Figure 3–5. Quantitative trait loci (QTL) associated with salt injury score (SIS) identified by Composite Interval Mapping (CIM) using the subset of Nested Association Mapping (NAM) population.

Table 3–6. Candidate genes within a range of 50 kb upstream and downstream to the linked marker of the identified QTL associated with salinity tolerance.

QTL	Species	Common name	Description	Identity Similarity	E−value	Accession
QSIS.kn17.2B	*Setaria italica*	Foxtail millet	Uncharacterized	87.5	0.00	XM_012843862.2
QSIS.kn17.9B	*Setaria italica*	Foxtail millet	Polyamine oxidase	93	$1e^{-166}$	XM_004985311.3
QSIS.kn18.6B	*Setaria italica*	Foxtail millet	Serine/arginine−rich splicing factor SR45a	85	$1e^{-122}$	XM_022827521.1
QSIS.kn19.2B	*Zea mays*	Maize	Uncharacterized	90.9	0.00	NM_001138406.1
QSIS.kn19.7B	*Sorghum bicolor*	Sorghum	SH3 domain−containing protein 2	95	$2e^{-83}$	XM_021464061.1
QSIS.kn.6A	*Setaria italica*	Foxtail millet	Non−specific lipid−transfer protein C4	90	$2e^{-100}$	XM_004974021.3
QSIS.kn.9B	*Setaria italica*	Foxtail millet	Dehydrodolichyl diphosphate synthase complex subunit NUS1	86	$9e^{-186}$	XM_022823027.1

CONCLUSION

Switchgrass (*Panicum virgatum* L.) is a warm−season perennial grass which assimilates carbon via the C4 photosynthetic pathway. Historically, switchgrass has been utilized in prairie renovation, wildlife conservation, erosion control, and forage for livestock. More recently, switchgrass has emerged as a bioenergy feedstock. Notable progress has been made in the past to improve biomass yield and forage quality through breeding. Although genetics underlying feedstock quality, stand stability under changing harvest management, and production of switchgrass on marginal lands such as salt-affected land is not well understood. Thorough understanding of these areas in switchgrass would help to improve biomass production and feedstock quality by implementing suitable breeding strategies. For this, three studies were conducted: (i) Genetic variation for biomass yield and predicted genetic gain in lowland switchgrass 'Kanlow', (ii) Assessment of genetic variation and identification of QTL associated with regrowth vigor in lowland switchgrass, and (iii) Identification of genomic regions associated with salinity tolerance in lowland switchgrass.

The first research is conducted with the objectives to assess genetic variation for biomass yield and the components of lignocelluloses, estimate the narrow sense heritability, and examine if phenotypic selection provides any significant gain in biomass yield in the KHS population. Switchgrass cultivar 'Kanlow' was chosen for this study because of two main reasons. First, it has great potential to use in breeding for an increased level of cold tolerance. Second, a very limited study was conducted in the past for the improvement of Kanlow population as compared to Alamo. Our results revealed significant genetic variation for biomass yield ($p < 0.05$) including feedstock quality traits hemi−cellulose content ($p < 0.05$), and lignin content ($p < 0.01$). The genotype × environment interactions had a great influence on biomass production ($p < 0.05$). Narrow sense heritability estimates for biomass yield was very low (0.10) compared to

quality traits hemi−cellulose (0.32) and lignin (0.66). These results suggest a potential challenge for biomass yield improvement. The magnitude of additive variance is small, and heritability is low for biomass yield, which illustrates that accumulation of favorable additive genes to improve biomass yield could be practiced by employing rigorous family−performance−based selection or among−and−within the family selection as suggested by Casler and Brummer (2008). Further, mean biomass yield of KHS did not differ from Kanlow control demonstrating that phenotypic selection would not be an efficient method to change the population mean in Kanlow. Because genotype × location interactions had a great influence on biomass yield, the cultivar development should be targeted according to the hardiness zone.

Stability of biomass yield and regrowth vigor under changing harvest management would help to deal with potential fluctuations in feedstock market and provide a continuous supply of quality forage for livestock. Past studies in other species suggested that regrowth after overwintering is a polygenic inherited trait (Paterson et al., 1995; Tao et al., 2008). Identification of genetic markers and application of marker−assisted selection would have a great impact on the improvement of quantitative traits (Collard and Mackill, 2008). To date, little is known on the underlying genetics associated with the response of a switchgrass plant to multiple cutting. No study has been exclusively conducted to identify QTL associated with regrowth vigor, especially after the increasing number of cuttings. Therefore, the second research project was conducted to adopt molecular breeding strategies by identifying QTL associated with regrowth vigor after multiple cutting in lowland switchgrass using a NAM population. A total of 10 QTL were detected, which accounted for phenotypic variation from 1.6% to 4.7% and the additive genetic effects ranged from −0.13 to 0.26. No single QTL showed a markedly large effect suggesting complex genetics underlying regrowth vigor in switchgrass. The majority of QTL associated

with regrowth vigor showed small additive effects, but strong dominance effects suggesting that these QTL have a heterozygous advantage. Therefore, the heterosis breeding scheme would be effective to produce the cultivar possessing highly vigorous regrowth. The transcript sequences of the identified QTL have a very high similarity with the genes identified in closely related species of switchgrass. Those genes are known to play a variety of roles in the developmental processes including plant hormonal signal transduction, nucleotide biosynthesis, secondary metabolism, senescence, and responses to both biotic and abiotic stresses. These functions provide strong evidence for the reliability of the identified QTL, which could be employed in MAS after validating their functional effect.

The third research was very similar to the second, which utilized a subset of NAM population to identify significant genomic regions associated with salinity tolerance in lowland switchgrass. Because salinity tolerance is genetically and physiologically complex (Koyama et al., 2001), the use of molecular markers would enhance cultivar breeding efficiency (Peleman and Van der Voort; 2003). To date, no published study is available on quantitative trait loci (QTL) mapping for salinity tolerance in switchgrass. In this study, seven QTL associated with SIS were detected, which accounted for 1.4% to 6.5% of the phenotypic variation. The additive genetic effects of an individual QTL ranged from −0.07 to 0.63. No single QTL having a large effect was detected for SIS. Nevertheless, this study is one step forward for improvement of salinity tolerance in switchgrass by implementing molecular breeding techniques. The markers linked to QTL identified in this study would be useful for gene pyramiding, and marker−assisted selection. Three candidate genes were identified, which may have a direct influence on salinity tolerance. However, an in-depth study is needed to validate the function of these candidate genes in switchgrass.

Overall, the results from this research would be beneficial to implement both classical and molecular breeding strategies to expedite the improvement of biomass yield, feedstock quality, stand stability, and salinity tolerance in switchgrass. Additionally, the knowledge gained about the additive and dominant genetic effect of the traits considered in this study would be helpful to adopt the most efficient breeding strategies. Further study is needed to understand the functional effect of the identified QTL that would play a vital role in the improvement of switchgrass.

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VITA

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He joined the University of Tennessee, Knoxville in 2014 to work for the switchgrass breeding program as a research associate. While working as a full−time staff, he enrolled as a part time PhD student in the fall 2015 and completed the degree in the fall 2019 with a major in plant breeding and genetics and a minor in statistics.

Santosh is very passionate about plant breeding. His career goal is to work as a breeder to contribute in the improvement of plants that would be beneficial to humankind.