Little Bluestem (*Schizachyrium scoparium*) and *Vitex*: Tissue Culture, Genetic Diversity, and Pollinators

by

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ABSTRACT

Native grasses are increasingly used in the landscape. Little bluestem (*Schizachyrium scoparium* L.), a native perennial bunchgrass, has good ornamental traits. Our objective was to develop an improved micropropagation protocol for little bluestem. In 2016, we cultured immature inflorescences of eight genotypes of little bluestem on MS medium with kinetin and 2,4-D under three levels of light to initiate callus. Level of kinetin had an effect on the induction of callus and number of rooted plants. Light and 2,4-D levels had no effect. In 2017, we cultured immature inflorescences of four genotypes of little bluestem on MS medium with 2,4-D and either kinetin or BAP. Cultures on medium with BAP had higher levels of callus induction than those on kinetin and produced more rooted plants.

Genetic and phenotypic variability in little bluestem makes it a good candidate for breeding new cultivars. Our objective was to assess genetic diversity among little bluestem genotypes from three regions in the United States: the Midwest, New England, and Georgia. We assessed genetic diversity of 49 genotypes of little bluestem using 10 polymorphic microsatellite markers that had been developed for the little bluestem cultivar 'The Blues'. An AMOVA revealed that 13.1% of the variation was among regions and 86.9% of the variation was within

regions. Nei's genetic distance was highest between the Midwest and Georgia groups and smallest between the New England and Midwest groups. Cluster analysis in Structure showed three groups. Regional groups did not cluster together.

Ten mature genotypes of *Vitex* L., an ornamental tree, were evaluated during summer 2016 to assess their attractiveness to pollinators. Pollinator counts were taken at 9:00 and 11:00 a.m. twice weekly for three weeks. Insects captured on *Vitex* plants and in the field were identified to genus. Bumblebees were further identified to species. Pollinators captured on the *Vitex* plants were principally bumblebees and honeybees. *V. agnus-castus* L. plants attracted more native bees than honeybees, while *V. negundo* L. plants attracted more honeybees than native bees. Our study shows that *Vitex* plants can be a good resource to support pollinators in an urban landscape.

INDEX WORDS: *Schizachyrium*, little bluestem, *Vitex*, micropropagation, microsatellites, native plants, urban landscapes, bumblebees, *Bombus*, pollinators

LITTLE BLUESTEM (*SCHIZACHYRIUM SCOPARIUM*) AND *VITEX*: TISSUE CULTURE, GENETIC DIVERSITY, AND POLLINATORS

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DEDICATION

For my son Brandon, always.

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CHAPTER 1

Introduction and Literature Review

Little bluestem [*Schizachyrium scoparium* (Michx.) Nash]

Little bluestem [*Schizachyrium scoparium* (Michx.) Nash] is a perennial, native warm-season (C_4) bunchgrass species (Fu et al., 2004). The plant is long-lived (Williams and Briske, 1991) and the species is widely adapted to the continental United States, with a range that extends from northern Mexico to southern Canada (Springer, 2012). Little bluestem has a high degree of drought tolerance and has been shown to be well adapted to dry areas (Boe and Bortnem, 2009). Little bluestem has been found to have associations with vesicular-arbuscular mycorrhizae (Anderson et al., 1984). The growth habit and leaf color of little bluestem are highly variable (Boe and Bortnem, 2009; Springer, 2012). Little bluestem has good fall color and visually interesting seed heads (Cullina, 2007).

Although little bluestem is a tough plant, with adaptability to a wide range of environments, it is not without a few problems with pests and disease. Cultivars released to date have been found to be highly susceptible to damage by two-lined spittlebugs [*Prosapia binacta* (Say.)] (Robacker, personal communication, 2014). However, the amount of spittle bug infestation appears to be highly dependent upon environmental variables, such as rainfall amount (personal observation). Little bluestem is also susceptible to leaf rust [*Puccinia andropogonis* (Schwein.)], a pathogen that is often found in native tallgrass prairies (Barnes et al., 2005). A study of 19 accessions of little bluestem found that all but two were very susceptible to the pathogen (Springer, 2012). Once infected, most accessions showed symptoms of the pathogen

throughout the entire plant (Springer, 2012). The disease not only causes the infected plants to become unsightly but also reduces photosynthetic capacity and production of biomass (Mitchell, 2003). A study of big bluestem found no genetic resistance to rust in the accessions tested (Barnes et al., 2005), yet testing additional to that of Springer et al. (2012) might find more genotypes of little bluestem with rust-resistance.

Little bluestem genetics

Little bluestem is a tetraploid (2n = 4x = 40) (Church, 1940; Springer, 2012). Examination of meiosis in the closely related big bluestem *(Andropogon gerardii* Vitman.), a hexaploid, found that normal bivalent pairing occurred during meiosis (Riley and Vogel, 1982). A meiotic study of little bluestem by Dewald and Jalal (1974) found univalents and lagging chromosomes were found in some of the microspores they examined. Little bluestem is considered to be a segmental allotetraploid with incomplete diploidization and insignificant effect on fertility by meiotic irregularities (Dewald and Jalal, 1974). Little bluestem readily outcrosses, and selfing will cause varying rates of inbreeding depression, depending on the genetics of the plant being selfed (Anderson, 1940). Breeding and selection have long been used effectively to produce improved cultivars of little bluestem, although early efforts were directed to producing better forage plants (Anderson and Aldous, 1938).

Genetic variability has been found both within and among populations of little bluestem. A study using AFLP markers found the most substantial part (91.5% to 92.8%) of the genetic variation of six populations of little bluestem from Manitoba, Canada was within populations (Fu et al., 2004). A study using RAPD markers on populations of little bluestem from grassland and forest ecosystems also found that most of the genetic variation was within populations, but that significant variation also existed among populations from each type of ecosystem (Huff et al.,

1998). Microsatellite markers (SSRs) have been developed for little bluestem using the cultivar 'The Blues'; and genetic diversity was assessed among accessions from the USDA-Agricultural Research Service (ARS) National Plant Germplasm System (NPGS) collection (Harris-Shultz et al., 2015).

Uses of little bluestem

Little bluestem has long been used as a forage grass and for restoration of natural areas. Little bluestem, along with big bluestem (*Andropogon gerardii* Vitman) and Indiangrass (*Sorghastrum nutans* (L.) Nash), are major species of tall-grass prairies in the continental United States (Chen and Boe, 1988). Little bluestem has been used in restoration of grasslands and both short-leaf pine and oak savannas (Brawn, 2006; Maynard and Brewer, 2013; Tober and Jensen, 2013). As a deep-rooted plant with adaptability to a variety of sites, little bluestem can be effective for erosion control (Tober and Jensen, 2013). Interestingly, cultivars of little bluestem such as Aldous and Camper showed higher water use efficiency (WUE) and net photosynthesis than did unimproved little bluestem used for prairie restoration projects (Lambert et al., 2011).

Little bluestem may be useful for restoring other areas than prairies. Creeping bluestem (*Schizachyrium stoloniferum* Nash), formerly known as *Schizachyrium scoparium* var. *stoloniferum*, has been grown on sand tailings from Florida phosphate mines with good survival and has been proposed as a useful plant for reclamation of mined lands (Kalmbacher et al., 2004). Little bluestem has shown a moderate degree of tolerance to salinity (Gibson and Carrington, 2008). Little bluestem, when planted with other prairie grasses such as big bluestem, has shown evidence of being able to remove polynuclear aromatic hydrocarbons (PAH) from soil, and thus may be useful in remediating oil spills on land (Aprill and Sims, 1990).

Little bluestem seeds are food for small mammals and birds (Tober and Jensen, 2013). Birds and small animals also use little bluestem for shelter (Tober and Jensen, 2013). Little bluestem provides nesting areas for ground-nesting birds such as the greater and lesser prairie chickens ([*Tympanuchus cupido pinnatus* (Brewster, 1885)] and [*T. pallidincinctus* (Ridgway, 1873)]) and quail [(*Colinus* ssp. (Goldfuss, 1830)] (Brawn, 2006; Maynard and Brewer, 2013; Robert, 1963; Tober and Jensen, 2013). Dusky skipper moths ([*Amblyscirtes alternata* (Grote and Robinson)]) use little bluestem as a host plant (Tober and Jensen, 2013).

As well as being used as a forage plant and for restoration of native habitats, cultivars of little bluestem have been used as ornamentals in the landscape (Fu et al., 2004). 'Cimarron' little bluestem has been used as part of the rough on golf courses (Maddox et al., 2007). Ornamental grasses, such as little bluestem, are an increasingly popular feature of home landscapes (Meyer, 2012). Sales of ornamental grasses increased by over 33 million dollars between 2009 and 2014 (https://quickstats.nass.usda.gov, accessed 17 January 2018). Higher sales of ornamental grasses will lead to demand for new cultivars of little bluestem. Many of the ornamental little bluestem cultivars on the market, such as 'Blue Heaven' and 'Carousel', were developed in the Midwest from plants of Midwestern provenance (Boehm, 2010; Meyer, 2006). Some cultivars developed in the Midwest do not perform well in the southeastern United States (personal observation). As the ornamental grass market grows, so will a need for regionally adapted varieties and new germplasm to incorporate into breeding programs.

Micropropagation

Hand in hand with developing new ornamental cultivars is the need to develop improved methods of clonally propagating the cultivars for dissemination to the public. Traditionally the method used has been division of stock plants (Meyer, 2012). However, the number of new

plants that may be produced by division is greatly limited by the availability of stock plants (Robacker and Corley, 1992). Micropropagation is a more efficient way of producing large numbers of true-to-type plants.

Ornamental grasses have previously been propagated through tissue culture. Immature inflorescences of pampas grass (*Cortaderia selloana* Schult. 'Pumila') were cultured on Murashige and Skoog (MS) medium with 2,4-Dichlorophenoxyacetic acid (2,4-D) and 6-benzyladenine (BA) to produce new plants (Robacker and Corley, 1992). Both shoot apices and immature inflorescences of *Miscanthus* x *giganteus* 'Freedom' were used to produce calli and stimulate shoots on MS medium with BA (Perera et al., 2015). Creeping bluestem was cultured on MS medium with 2,4-D and various combinations of BA, abscisic acid (ABA), gibberellic acid (GA_3), and zeatin to provide plants for remediation of phosphate-mined land (Chakravarty, 2001). Big bluestem was cultured from the rachis of immature inflorescences in a dark environment on media containing 5 mg•L^{-1} 2,4-D and either with or without kinetin (Chen et al., 1977). Mature seeds of big bluestem on MS medium combining BA and 2,4-D were used to induce callus, and shoots were then generated from the calli on MS medium with BA or kinetin (Pantha, 2016).

Mature caryopses cultured under dark conditions on Linsmaier and Skoog (LS) medium with kinetin and 2,4-D were used to induce callus in both big bluestem and little bluestem (Li et al., 2009). Shoot regeneration was then performed by transferring the calli to medium with kinetin and plantlets were transferred to medium with 1-Naphthaleneacetic acid (NAA) to induce root formation (Li et al., 2009). Immature inflorescences of little bluestem cultured on revised MS (RM) medium with 2,4-D and NAA and incubated in the dark were used to produce calli (Songstad et al., 1986). Calli was then subcultured for several rounds on medium with kinetin

and then incubated in the light on medium with no additional hormones to produce shoots (Songstad et al., 1986). Current micropropagation protocols for little bluestem could be improved to provide a rapid way of producing large numbers of true-to-type plants.

Vitex

The genus *Vitex* is the largest in the Verbenaceae family (Rani and Sharma, 2013). Species in *Vitex* are distributed in Asia, India, the Mediterranean, Pakistan, Sri Lanka, and southern Europe (Rani and Sharma, 2013). *Vitex* species are predominantly deciduous shrubs, but some species many grow to the size of a large tree (Rani and Sharma, 2013).

Morphology of *Vitex* species is as diverse as their origins. *Vitex agnus-castus* Linn. is deciduous, has floral displays that attract many pollinators, and may be a somewhat sprawling large shrub or a small tree (Rani and Sharma, 2013). *Vitex negundo* Linn. is a large deciduous shrub with an erect growth habit and many purple flowers (Rani and Sharma, 2013). *Vitex rotundifolia* Linn. is a sprawling deciduous shrub reaching approximately two feet tall and having small clusters of purple flowers (Cousins et al., 2010; Rani and Sharma, 2013). *Vitex trifolia* L. is a large shrub with either trifoliate or simple leaves with panicles of blue or lavender flowers (Ahmed et al., 2015).

Although generally a robust plant, some species and accessions of *Vitex* have proven susceptible to disease. Corynospora leaf spot has been reported to occur on beach vitex (*V. rotundifolia*) in Korea (Park et al., 2013). Cercospora leaf spot has been found to affect *V. agnus-castus*, *V. rotundifolia*, and hybrids of the two species (Hershberger et al., 2010). An African species of *Vitex*, *V. doniana*, is susceptible to rust (Kapooria and Aime, 2005). *Vitex* hybrids in Griffin, GA, have been observed to be susceptible to an unknown leaf spot disease, which is possibly bacterial in origin (Personal observation, 2015; Martinez, personal communication,

2015; Robacker, personal communication, 2015). Any *Vitex* cultivar that is developed must be carefully screened for disease susceptibility, as unsightly leaf spots will render plants unmarketable.

Cold-hardiness is an issue for *Vitex* in the northern United States. *Vitex* species are considered to be cold-hardy up to USDA zone 6 (Harrison, 2009). *V. agnus-castus* 'Silver Spires' did not survive the winter in cold-hardiness trials in Maine (Capiello and Littlefield, 1994). A cold-hardiness trial of *V. agnus-castus*, *V. cannabifolia* Siebold & Zucc., and *V. negundo* in the Ukraine found that *V. negundo* and *V. cannabifolia* had more cold-hardiness than *V. agnus-castus* (Nataliia and Volodymyr, 2018). Possibly cultivars of *V. negundo*, or hybridization between *V. agnus-castus* and *V. negundo* hybrids, might extend the range of ornamental *Vitex* northward.

Vitex cultivars must also be screened for invasive potential. *V. rotundifolia* was introduced to the coastal regions of the Carolinas to prevent beach erosion but quickly became a threat to the dune ecosystems due to its invasiveness (Cousins et al., 2010). *V. rotundifolia* has also naturalized in several other southeastern states (Cousins et al., 2010). *V. agnus-castus* has shown a tendency to naturalize in parts of Florida and has been listed as invasive in Texas (Judd, 2003; http://www.texasinvasives.org/plant_database/detail.php?symbol=VIAG, accessed 10 Jul 2015). The Center for Invasive Species and Ecosystem Help (www.invasive.org, accessed 10 Jul 2015) lists *V. trifolia* as an invasive plant in Florida. Since invasive plants have an economic as well as ecological effect, responsible plant breeding requires that potential cultivars be tested for invasive potential and, if possible, be bred for sterility. Interspecific hybrids often have sterility issues (Eeckhaut et al., 2007; Reed et al., 2008). However, sterility cannot be assumed but must be thoroughly tested.

Vitex genetics

V. negundo is a diploid whose chromosome counts have been reported variously as 2n = 24, 26, 32, or 34 (de Kok, 2007; Malik and Ahmad, 1963; Santosh and Gupta, 2012; Sobti and Singh, 1961). *V. agnus-castus* has a chromosome count of 2n = 24 (Darlington et al., 1955). *V. trifolia* has been reported to have a chromosome count of 2n = 26 or 2n = 32 (Ahmed et al., 2015; Sobti and Singh, 1961). Despite varying chromosome counts, interspecific hybrids from crosses between *Vitex* species have been achieved. In the Robacker breeding program, one such cross is an interspecific hybrid between *V. rotundifolia* and *V. agnus-castus* 'Shoal Creek'. The plant, accession number V0502-7 in the breeding program, has large and attractive inflorescences of purple flowers but an undesirable sprawling growth habit. Attempts to use V0502-7 in further rounds of breeding have been stymied by the sterility of the plant.

Although *Vitex* species have been propagated from seeds and cuttings, as well as through micropropagation (Rafique and Mohammad, 2014; Rahman and Bhadra, 2011), to our knowledge neither embryo rescue nor ovule culture have been used for propagation. In many species, seed of interspecific crosses are often difficult to germinate, and embryo rescue has been used to successfully propagate hybrids. Ovule culture was used to rescue interspecific hybrids of *Abelia* x *grandiflora* (André) Rehd. 'Francis Mason' x *A. schumanii* (Graebn.) Rehd. (Scheiber and Robacker, 2003). Interspecific *Rhododendron* hybrids were also rescued by ovule culture, although the degree of success greatly depended on which cultivar was the pollen parent (Eeckhaut et al., 2007). An interspecific cross between *Viburnum lantana* 'Mohican' and *V. carlesii* 'Aurora' would only produce viable progeny when embryo rescue was used (Hoch et al., 1995). An even wider cross of *Dichroa febrifuga* (Lour.) and *Hydrangea macrophylla* (Thunb.) produced no seedlings except through ovule culture (Reed et al., 2008). To our knowledge,

neither embryo rescue nor ovule culture has been attempted for germination of interspecific *Vitex* hybrids, even though germination is poor for certain interspecific combinations (Robacker, personal communication, 2014).

Vitex uses

Many people know of *Vitex* primarily through its reputation as a medicinal plant. *V. negundo*, called nirgundi in India, is an important plant in Ayurvedic medicine where it is used as an anthelmintic (Ahuja et al., 2015). *V. agnus-castus* has long been used to treat reproductive imbalances in women (Padmalatha et al., 2009). *V. trifolia* has anti-bacterial activity (Padmalatha et al., 2009).

However, species of *Vitex* have many other uses than medicinal. Branches of *V. negundo* have been used in basketry, and leaves are used as an insect repellent (Ahuja et al., 2015). *V. agnus-castus* showed promise as a trap crop for the planthopper *Hyalesthes obsoletus* Signoret (Homoptera: Cixiidae) in vineyards in Israel (Sharon et al., 2005). Extracts of *V. trifolia* have been shown to have larvicidal properties against mosquito species *Culex quinquefasciatus* (Wiedemann, 1828) and *Aedes aegypti* (Linnaeus, 1762) and have been investigated as a control for mosquitoes (Kannathasan et al., 2011). Clearly, it would be a mistake to pigeonhole *Vitex* species as a purely medicinal plant.

V. negundo has shown moderate tolerance to air pollution (Liu and Ding, 2008; Sumangala et al., 2018). *V. agnus-castus* showed moderate salt tolerance and high biomass production in a study of the effect of treated wastewater on plant species to be used in restoration of riparian areas (Adrover et al., 2008). *V. negundo* L. var. *cannabifolium* was found to accumulate a high level of lead (Pb) in a study of three sites in China contaminated with heavy metals (Peng et al., 2006). *V. negundo* var. *heterophylla* accumulated high levels of zinc (Zn) in

a study at a Chinese mine (Zhao et al., 2014). *V. trifolia* var. *simplicifolia* accumulated high levels of copper (Cu), lead, and zinc in a trial of plant species as bioaccumulators (Shi et al., 2011). *Vitex* species are well-adapted to be urban ornamental plants and have possible uses in bioremediation.

Vitex species have a long history of being attractive to pollinators. *Vitex agnus-castus* and *V. negundo* have been used as honey plants (Dogan et al., 2011; Harugade et al., 2016). *V. negundo* is considered an important source of pollen for bees in Korea (Jung and Lee, 2018). Flowers of *Vitex* species have been shown to attract to a wide range of pollinators, including bumblebees, honeybees, and butterflies (Ashoke and Sudhendu, 2012; Jain, 2013; Murren, 2014; Reddy et al., 1992). The inclusion of *V. negundo* had a positive effect on bee abundance in a study that included several types of landscapes in China (Wu et al., 2018).

Vitex and Pollinators in the Urban Landscape

Although pollinators such as bumblebees and honeybees are important for the production of agricultural crops (Koh et al., 2016), they also serve important ecological functions. Pollinators, including wild bees, pollinate 87.5% of all flowering species worldwide (Ollerton et al., 2011). Unfortunately, pollinators have faced many challenges in recent years. Increasing urbanization and conversion of former pollinator habitat to agricultural uses leads to loss of habitat for pollinators (Potts et al., 2010). Loss of habitat can lead to decline in both population and species richness of pollinators (Brown and Paxton, 2009; Goulson et al., 2015; Hernandez et al., 2009). Add exposures to pesticides, pathogens, and pests (Cameron et al., 2011; Goulson et al., 2015), and pollinators face formidable challenges indeed.

Urban landscapes that are designed to mitigate habitat loss may help pollinators face these challenges. Not only have urban areas been shown to have higher populations of bees than

agricultural areas, but also bees in urban areas have been shown to have stored more food and have higher reproductive fitness than those in agricultural areas (Senapathi et al., 2017; Samuelson et. al, 2018). Urban areas surrounded by agricultural land might even serve as a refuge for bee populations (Samuelson et al., 2018).

However, the question of which plants to use in urban landscapes to support pollinators, especially native pollinators, remains. Many landscape plants are non-native. Non-native plants have been shown to be detrimental to native pollinators, possibly by providing less nutrition (Wilde et al., 2015). Pollinator species abundance was four times less on non-native plants than on native plants in a study in Pennsylvania (Burghardt et al., 2009). However, any detriment to non-native plants that affect pollinators may be due more to changes in floral morphology than native status. Studies of garden flowers in Britain found that native status of a plant had less effect on attractiveness of its flowers than did morphological alterations, such as double flowers, made through plant breeding (Corbet et al., 2001; Garbuzov and Ratnieks, 2014). In a landscape with few floral resources, such as many urban and suburban areas, non-native plants have been shown to provide much needed resources for pollinators (Stout and Morales, 2009). Non-native species have been show to provide ecological services, such as shelter for wildlife and food for pollinators (Schlaepfer et al., 2011). As the world becomes more urban, a need for landscape plants that are adapted to urban areas and can provide ecological services will be increasingly necessary. Although not a native to the United States, *Vitex* may still be desirable in the urban landscape as a plant for pollinators.

Schizachyrium Research Objectives

Previous studies of micropropagation in little bluestem have involved numerous rounds of callus induction, increasing the time needed to produce rooted plants and increasing the

possibility of somaclonal variation in the plants. Some previous studies also used as explants mature caryopses, which meant that plants produced would be genetically different from the source plant. Our objective in this study was to develop an improved micropropagation protocol for little bluestem that would produce true-to-type plants. To do this, we sought to minimize the time spent culturing callus. In addition, we used immature inflorescences as our explant, in order to preserve the genotype of the source plants.

While several studies of genetic diversity in little bluestem have been done, genotypes in those studies were predominantly from the midwestern area of the United States and in Canada. To our knowledge, no studies of genetic diversity in little bluestem from New England or the southeastern United States have been examined. Our objective was to investigate the genetic diversity of little bluestem genotypes from New England, Georgia, and the midwestern United States using SSR markers developed by Harris-Shultz et al. (2015).

Vitex Research Objectives

Ornamental plant cultivars must fulfill many requirements. Cultivars must not only be attractive and low-maintenance but also adaptable to harsh urban conditions. In addition, there is increasingly a call for ornamental plant varieties to fulfill ecological functions. One important function is to provide services for pollinators, especially native pollinators such as bumblebees. Our primary research objective for *Vitex* was to evaluate the attractiveness of *Vitex* to pollinators. We are especially interested in knowing if native pollinators are attracted to *Vitex*.

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CHAPTER 2

Micropropagation of Little Bluestem (*Schizachyrium scoparium* L.)[1]

[1] Hawkins, S.M. and C. Robacker. To be submitted to *HortScience*.

Abstract

Native grasses are increasingly used in the landscape. Little bluestem (*Schizacyrium scoparium* L.), a perennial bunchgrass native to most of the United States, has good ornamental traits, such as variation in leaf color, differences in growth morphology, and attractive seed heads. Traditionally cultivars of little bluestem are propagated by division, which limits the production of new plants. Our objective in this study was to develop an improved micropropagation protocol for little bluestem that would produce true-to-type plants. In 2016, we cultured immature inflorescences of eight genotypes of little bluestem on MS medium with four combinations of kinetin (1.0 or 2.0 mg•L^{-1}) and 2,4-D (0.5 or 1.0 mg•L^{-1}) under three levels of light (dark, semi-light, full light) to initiate callus. Cultures were evaluated 30 days after initiation and those that had initiated callus were subcultured. Media for subculturing and rooting either contained 0.1 mg•L^{-1} or no NAA. Light level had no effect on callus initiation ($p = 0.1360$). Initiation media with 1.0 mg•L^{-1} kinetin and either level of 2,4-D induced callus at highest rates ($p = 0.0002$), and cultures initiated on those media produced the highest number of rooted plants over all genotypes ($p = 0.0410$). Genotype affected the number of rooted plants produced ($p = 0.0187$). The addition of NAA to medium for subculturing and rooting did not increase the number of rooted plants ($p = 0.1081$). In 2017, we cultured immature inflorescences of four genotypes of little bluestem on MS medium with 0.5 mg•L^{-1} 2,4-D and 1.0 mg•L^{-1} and either kinetin or BAP under full light. Cultures were evaluated 30 days after initiation. Cultures that had initiated callus were subcultured onto MS medium with the same growth regulators as the initiation media but without 2,4-D. Cultures were cycled between subculture medium with growth regulator and subculture medium with no additional growth regulator until rooted. Cultures initiated and subcultured on medium with BAP had higher levels of callus induction

than those on kinetin ($p < 0.0001$) and produced more rooted plants ($p = 0.0004$). Our recommendation for rapid micropropagation of little bluestem is to initiate cultures on MS medium with 1.0 mg•L^{-1} BAP and 0.5 mg•L^{-1} 2,4-D. After callus initiation, cultures should be subcultured to medium with BAP but no 2,4-D, alternating with medium with no additional growth regulators, until rooted.

Ornamental grasses are increasingly utilized in the landscape (Meyer, 2012). Sales of ornamental grasses in the United States were $158,061,021 in 2014, a substantial increase from $124,261,118 in 2009 (https://quickstats.nass.usda.gov, accessed 17 January 2018). As well as being attractive, native ornamental grasses provide ecological functions such as food and nesting areas for wildlife and support for pollinators (Fu et al., 2004; Tober and Jensen, 2013). Native species of ornamental grasses may also provide drought-tolerance and adaptability to local landscapes (Meyer, 2012).

Little bluestem (*Schizachyrium scoparium* L.) is a perennial grass native to most of the continental United States (Fu et al., 2004; Springer, 2012; Williams and Briske, 1991). In recent years, it has also become a popular landscape plant (Fu et al., 2004; Meyer, 2012). Little bluestem has a wide range of adaptability to climate and soils and is drought-tolerant (Meyer et al., 2017; Springer, 2012; Tober and Jensen, 2013). Little bluestem possesses many good ornamental traits, such as variation in leaf color and growth habit, as well as attractive seed heads (Boe and Bortnem, 2009; Cullina, 2007; Springer, 2012).

Most ornamental grasses, like other ornamental perennial plants, are clonally propagated (Meyer et al., 2017). Often the method that growers use is division of stock plants (Meyer, 2012). However, the number of new plants is limited by the number of stock plants to be divided (Robacker and Corley, 1992). Micropropagation is a more effective method of propagation to obtain large numbers of new plants.

Micropropagation has been used to propagate ornamental grass species such as pampas grass and miscanthus. Shoot apices and immature inflorescences of *Miscanthus* x *giganteus* ‘Freedom’ were used to produce calli and regenerate shoots on Murashige and Skoog (MS) medium with 6-benzyladenine (BA) (Perera et al., 2015). Pampas grass (*Cortaderia selloana*

Schult. ‘Pumila’) was propagated through tissue culture using immature inflorescences on MS medium with 2,4-Dichlorophenoxyacetic acid (2,4-D) and BA (Robacker and Corley, 1992). Chen et al. (1977) cultured big bluestem (*Andropogon gerardii* Vitman) from the rachis of immature inflorescences on media containing 5 mg•L^{-1} 2,4-D and either with or without kinetin under dark conditions. However, Pantha et al. (2016) induced callus from mature seeds of big bluestem on MS medium combining BA and 2,4-D (Pantha et al., 2016). Shoots were subsequently generated from the calli on MS medium with BA or kinetin (Panthaet al., 2016). Callus was induced in both big bluestem and little bluestem by culturing mature caryopses under dark conditions on Linsmaier and Skoog (LS) medium with kinetin and 2,4-D (Li et al., 2009). Shoots were regenerated by transferring the calli to medium with kinetin; then the plantlets were transferred to medium with 1-Naphthaleneacetic acid (NAA) to induce root formation (Li et al., 2009). Calli were produced from immature inflorescences of little bluestem cultured on revised MS (RM) medium with 2,4-D and NAA and incubated in the dark (Songstad et al., 1986). The calli subsequently produced shoots after several rounds of subculturing on medium supplemented with kinetin followed by incubation under light conditions on hormone-free medium (Songstad et al., 1986).

Our objective in this study was to develop an improved micropropagation protocol for little bluestem that would produce true-to-type plants. To do this, we sought to minimize the time spent culturing callus, as longer times in callus can increase the chance of somaclonal variation (Neibaur et al., 2008). In addition, we used immature inflorescences as our explant, in order to preserve the genotype of the source plants.

Materials and Methods

Plant Material and Disinfestation

In 2016, immature inflorescences of eight genotypes of little bluestem were harvested from plants in the field plots of the joint breeding program between the University of Georgia and the USDA in Griffin, GA. Three genotypes were cultivars that had been released by the program and the remaining genotypes were selections from the program that were undergoing evaluation as potential new cultivars (Table 2.1). In 2017, immature inflorescences of four of the genotypes that had been used the previous year were harvested (Table 2.1). Inflorescences were harvested prior to the flag leaf becoming horizontal and when the entire bundle of sheath leaves enclosing the inflorescence was less than 7.5 to 9.0 cm long. The inflorescences that were harvested were fully enclosed in sheath leaves.

Outer leaves were partially removed from the inflorescences using a scalpel and tweezers and without exposing the inflorescence itself. The inflorescences, still enclosed in sheath leaves, were then washed for 10 minutes in soapy water, rinsed in running water for 10 minutes, soaked for 20 minutes in a 20% solution of commercial bleach, and rinsed 3 times in sterile distilled water. The remaining sheath leaves were removed under sterile conditions. Inflorescences no longer than 2.5 cm in length were selected for culture (Figure 2.1). Inflorescences were cut into pieces ≈ 5 to10 mm long to produce individual explants. Two explants were placed horizontally into each culture tube.

Culture treatments- 2016

Three light treatments were used during callus induction: full light provided by 110-W wide-spectrum fluorescent bulbs (70 $\mu mol \cdot m^{-2} \cdot s^{-1}$), semi-light (provided by placing the cultures in an opaque box with a translucent lid), and dark (provided by placing the cultures in a totally

opaque box). Average PAR measurements ($\mu mol \cdot m^{-2} \cdot s^{-1}$) at bench level were 36.98, 7.88, and 0.02 for full light, semi-light, and dark respectively.

Cultures were initiated on MS medium (Murashige and Skoog, 1962) and vitamins, 30.0 $g \bullet L^{-1}$ sucrose, and 0.75 $g \bullet L^{-1}$ $MgCl_2$. The pH was adjusted to 5.8 with 1 N NaOH and the medium was solidified with 2.0 $g \bullet L^{-1}$ Gelrite. Two growth regulators were added: 2,4-Dichlorophenoxyacetic acid **(**2,4-D) at either 0.5 or 1.0 $mg \bullet L^{-1}$ and kinetin at either 1.0 or 2.0 $mg \bullet L^{-1}$. The medium was dispensed in 8-ml aliquots into 25x150-mm culture tubes capped with clear Magenta caps (Magenta, Chicago, IL) and autoclaved for 20 minutes at 121°C. At least two explants were cultured per combination of genotype, media, and light treatment. Due to limitations of the plant material available to us and the number of factors being tested, we were not able to culture larger numbers of explants. Cultures were maintained at 27 to 30°C.

Cultures were scored 30 days after the initial culture date. A rating scale of 0 to 5 was used (Table 2.2). A score of 0 was given to cultures where the tissue had died. Cultures with a score of 1 or higher were subcultured after being rated.

Medium for subculturing and rooting was identical to the initiation medium with the exception of the growth regulators. The effect of NAA on rooting was tested. Cultures with live tissue were initially subcultured onto medium without NAA for further growth. The culture produced from the original explant was kept intact and transferred to an individual tube. After 30 days on medium without NAA, half of the cultures were subcultured onto medium with 0.1 $mg \bullet L^{-1}$ NAA (Sigma Chemical Co.) and the other half onto fresh medium without NAA. Cultures for this and subsequent subcultures were not kept intact but were broken into pieces. Cultures were subcultured every four to six weeks. Cultures that had been subcultured on

medium with NAA were cycled between the medium with NAA and medium without NAA until rooted. The remaining cultures were subcultured onto medium without NAA until rooted.

All pieces of cultures in a tube were placed onto fresh media during subculturing. Any rooted cultures were separated from unrooted cultures and placed in tubes with fresh media with no growth regulator to await transplantation. Cultures were initiated in July and August and were subcultured until January 2017.

Culture Treatments – 2017

In 2017, all cultures were initiated under 110-W wide-spectrum fluorescent lights. Initiation media and culture conditions were similar to that used in 2016 except for growth regulators. In 2017, the initiation medium included 0.5 mg•L^{-1} of 2,4-D and either 1.0 mg•L^{-1} kinetin or 1.0 mg•L^{-1} 6-benzylaminopurine (BAP). At least 60 explants were cultured per combination of genotype and media treatment. Cultures were scored 30 days after the initial culture date according to the scale in Table 2.2._Medium for subculturing and rooting was identical to the initiation medium with the exception of the growth regulators.

Cultures with live tissue were subcultured onto medium with either 1.0 mg•L^{-1} BAP or 1.0 mg•L^{-1} kinetin for further growth. Cultures were initially subcultured onto medium with the same cytokinin as the medium on which they had been initiated. As in 2016, the culture produced from the original explant was kept intact and transferred to an individual tube. In 2017, each original explant was assigned an individual number after the initial subculture, to track the rate of rooting. Cultures were subcultured every four to six weeks (Figure 2.2). Cultures were broken into pieces after the initial subculture and at every subculture tubes were labelled with the number given to the initial explant. From the second subculture on, cultures were cycled between medium containing cytokinin and medium with no cytokinin until rooted. All pieces of cultures

in a tube were placed onto fresh media during subculturing. Cultures were initiated in July and August and were subcultured until February 2018.

Transplantation and Acclimatization

We considered plants rooted when they had at least one root that was at least 1 cm in length. Number of days to rooting was measured as the number of days between initial culture date and the day the plant was transplanted to the greenhouse. Rooted plants were transferred to Sungro Professional Growing Mix (Sungro Professional Growing Mix, Sun Gro Horticulture, Agawam, MA) and acclimated under intermittent mist (10 s every 30 min) with no shade in the greenhouse for two to three weeks. Plants were maintained in the greenhouse once they had acclimated (Figure 2.3).

Data Analysis

A completely randomized design was used for both the 2016 and 2017 experiments. Data were analyzed in SAS 9.3 (SAS Institute, Inc., Cary, NC) using proc glimmix. Means separation was performed using Tukey's HSD ($P < 0.05$) treatments for differences within treatment method. Analyses for individual factors, such as initiation media or genotype, were done over all treatment combinations unless otherwise noted. Each explant was considered one replicate.

Results and Discussion

2016 Experiment

Initial light conditions had no effect on cultures in 2016 (data not shown). Although the scores of cultures rated 30 days after the initial culture date under light and semi-light conditions were higher than those under dark conditions, the differences were not significant ($p = 0.1360$). Initial light treatment also had no effect on days to rooting ($p = 0.7914$) or on the number of rooted plants obtained per initial culture ($p = 0.3222$).

Previous studies of micropropagation of little bluestem have incubated cultures in the dark to produce callus (Chen and Boe, 1988; Li et al., 2009; Songstad et al., 1986). Cultures in the Songstad et al. (1986) study were also incubated in the dark to produce shoots. However, our study shows that incubation in the dark is not necessary to produce either callus or shoots.

Initiation media made a clear difference in scores of cultures rated 30 days after the initial culture date. Cultures on initiation media with kinetin at 1.0 mg•L^{-1} and either 0.5 or 1.0 mg•L^{-1} 2,4-D had higher scores than cultures on kinetin at 2.0 mg•L^{-1} and either level of 2,4-D ($p = 0.0002$) (Table 2.3). The results we obtained using 2,4-D are contrary to the study of micropropagation of little bluestem by Songstad et al. (1986), who found that callus production was highest on medium with the highest concentration, 5 mg•L^{-1}, of 2,4-D. However, explants later transferred to medium with 0.1 mg•L^{-1} of 2,4-D produced fewer shoots than medium without 2,4-D (Songstad et al., 1986). Our results are also different than those of the study by Li et al. (2009) of micropropagation of mature caryopses of little bluestem. In that study, higher amounts of 2,4-D in the initiation media produced larger amounts of callus, especially when combined with kinetin (Li et al., 2009). The amount of 2,4-D may also have an effect on the rate at which callus is produced. In the study by Songstad et al. (1986), explants cultured on higher amounts of 2,4-D took eight weeks to produce enough callus to subculture (Songstad et al., 1986), while in our study we were able to produce callus after four weeks using lower amounts of 2,4-D combined with another growth regulator.

Our results using different concentrations of kinetin in initiation media are different from those of the study of micropropagation of little bluestem by Li et al. (2009), that showed no real difference in the amount of callus produced on initiation media with different amounts of kinetin. This is also contrary to results from the Songstad et al. (1986) study that found that cultures

transferred to media containing 2,4-D and higher amounts of kinetin produced more shoots. Our study shows that increased amounts of kinetin in initiation media are not necessary in order to produce callus and shoots.

The number of rooted plants obtained per initial culture was different among initiation media over all initial light conditions, subculture media, and genotypes ($p = 0.0410$) (Table 2.3). Cultures initiated on either of the media containing 1.0 mg•L^{-1} kinetin produced more rooted plants than media with 1.0 mg•L^{-1} 2,4-D and 2.0 mg•L^{-1} kinetin. In addition, initiation media significantly affected the number of days to rooting ($p = 0.0065$) from the initial culture date (Table 2.3), although cultures on all initiation and subculture media produced rooted plants in 110 to 122 days.

Genotype made a clear difference in the scores of cultures rated 30 days after the initial culture date ($p = 0.0187$, Table 2.4). In 2016, 'Good Vibrations', 'Seasons in the Sun', BX6-7 and BX8-4 had the highest scores. The number of rooted plants obtained per explant scored after 30 days and placed on subculture media was also different among genotypes ($p = 0.0187$) (Table 2.4). Genotype BX6-9 produced the highest number of rooted plants averaged over initiation media, initial light conditions, and subculture media. Due to mortality of some cultures, genotype B24-3 was not fully replicated across all treatments so it was removed from the analysis. Genotype also had an effect on days to rooting from initial culture date ($p < 0.0001$) (Table 2.4). Genotype BX6-9, at 101 days, was quickest to root. In contrast, 'Cinnamon Girl' and BX8-4 took 126 days to root from the initial culture.

Genotype has previously been shown to have an effect on micropropagation of grasses. In a study of micropropagation of three genotypes of little bluestem, one genotype had almost three times the number of shoots than another genotype (Songstad et al., 1986). Genotype had a

significant effect on the number of shoots regenerated in the micropropagation of 'Bison' and 'Bonilla' big bluestems, with 'Bonilla' producing almost twice as many shoots on the same media (Li et al., 2009). The percentage of callus induction of four genotypes of *Miscanthus lutarioriparius* L. Liou ex Renvoize & S. L. Chen ranged from 95% for two genotypes down to 15% for one genotype on identical media (Zhao et al., 2016).

NAA in subculture medium significantly affected the number of days to rooting from initial culture ($p = 0.0006$) (Table 2.5). Plants on media without NAA produced rooted plants 12 days before plants on media with 0.1 mg•L^{-1} NAA. However, NAA made no difference in the number of rooted plants obtained across all genotypes ($p = 0.1081$) (Table 2.5). Cultures subcultured on medium containing NAA produced an average of 1.5 plants per culture placed on rooting medium, while cultures subcultured on medium with no NAA produced an average of 2.2 plants over all genotypes, initial light treatments, and initiation media.

The addition of up to 0.5 mg•L^{-1} NAA had no effect on the rate of rooting of little bluestem cultures in a study by Li et al. (2009). Additionally, in that study, cultures rooted on media with 1.0 mg•L^{-1} or higher NAA produced significantly fewer roots. Pantha et al. (2016) reported a 90% rate of rooting on media with no additional hormones for big bluestem cultures. However, cultures of *Miscanthus sinensis* had higher percentages of rooting on media supplemented with 0.2 mg•L^{-1} NAA than on media with no additional NAA, although higher concentrations of NAA had a deleterious effect on rooting (Zhang et al., 2012). The value of NAA as an addition to media to promote rooting may be species dependent.

Mortality of plants rooted and transplanted to the greenhouse was 6.0%. Evaluation of plants in the greenhouse and in the field showed no morphological differences.

2017 Experiment

We used full light conditions for all cultures from initiation to rooting. As the concentration of 2,4-D had no effect in 2016, only 0.5 mg•L^{-1} 2,4-D was used in initiation media in 2017. Furthermore, since initiation media with 1.0 mg•L^{-1} kinetin produced more callus and shoots in 2016 than initiation media with 2.0 mg•L^{-1} kinetin, in 2017 we used the lower amount of kinetin in initiation media to compare with initiation media containing BAP. Adding NAA to subculture media in 2016 did not result in a greater number of rooted plants, and explants on subculture media with NAA produced roots more slowly. Therefore, NAA was not added to subculture media in 2017.

Scores of cultures rated 30 days after the initial culture date were different between initiation media. Cultures on medium with 1.0 mg•L^{-1} BAP had higher scores than cultures on medium with 1.0 mg•L^{-1} kinetin ($p < 0.0001$) (Table 2.3). The number of rooted plants obtained per explant cultured was different between media with BAP and media with kinetin over all genotypes ($p = 0.0004$) (Table 2.3) with BAP having a more than two-fold increase in number rooted per explant. Explants cultured on initiation and subculture media containing BAP produced more rooted plants than those cultured on initiation and subculture media containing kinetin.

Cytokinins are effective at stimulating shoot initiation in tissue culture (Thomas et al., 1996). Both BA and kinetin have been used as effective components in the micropropagation of grasses. Media supplemented with BA was more successful than media without BA in micropropagation of creeping bluestem (*Schizachyrium scoparium* (Michx.) Nash var. *Stoloniferum* (Nash) J. Wipff) (Chakravarty, 2001). When a moderate amount of BA was added to media with 2,4-D in the micropropagation of *Miscanthus sinensis* Andersson, higher amounts

of embryonic callus were produced (Zhang et al., 2012). Callus cultures of bermudagrass had enhanced ability to regenerate shoots when BA was added to media containing 2,4-D (Chaudhury and Qu, 2000). However, kinetin was more successful than BA in regenerating shoots during micropropagation of big bluestem (Pantha et al., 2016). Shoot regeneration was improved and shoot count increased with the addition of kinetin to media in other studies of micropropagation of little bluestem (Li et al., 2009; Songstad et al., 1986). To our knowledge, no other studies of micropropagation of little bluestem have compared the effects of incorporating BA versus kinetin into the media. In our study, although kinetin was an effective component of media for micropropagation of little bluestem, it was not as effective as BAP.

Initiation and subculture media also affected the number of days to produce rooted plants ($p < 0.0001$) (Table 2.3). Cultures initiated and subcultured on media with kinetin produced rooted plants more quickly than those on media with BAP. However, cultures on all initiation and subculture media produced rooted plants in 181 to 196 days.

Genotype made a clear difference in the scores of cultures rated 30 days after the initial culture date ($p < 0.0001$, Table 2.4). Genotypes BX6-7 and BX8-4 had the highest scores. A difference was also found among genotypes in the number of rooted plants obtained per explant scored and placed on subculture media ($p = 0.0012$) (Table 2.4). Explants of genotypes BX6-7 and BX6-9 produced more rooted plants than explants of genotypes ‘Seasons in the Sun’ and BX8-4. However, genotype had no effect on the number of days to rooting across all initiation and subculture media ($p = 0.2530$) (Table 2.4). All genotypes rooted in 181 to 197 days.

Mortality of plants rooted and transplanted to the greenhouse was 6.3% in 2017. Only two plants out of 1,590 transplanted showed morphological differences in 2017. Leaves of the

two plants with morphological differences were more yellow and thicker than those of the parent plant. All other little bluestem plants produced by micropropagation grew true-to-type.

Comparing 2016 to 2017

A comparison of scores 30 days after initiation across all genotypes and media in 2016 versus 2017 revealed that scores were higher in 2016 (Table 2.3). The higher percentage of cultures with a score of 0 (dead tissue) in 2017 than in 2016 lowered the average score of cultures initiated in 2017. In 2016, 38.8% of cultures initiated were given a score of 0, while in 2017 a score of 0 was given to 58.8% of cultures initiated. In 2017, three of the four genotypes in the study - 'Seasons in the Sun', BX6-7, and BX8-4 – bloomed later than in 2016 (7 days, 9 days, and 15 days respectively). For all genotypes in 2017, inflorescences were slower to develop to the optimal stage for culturing after plants were in bloom (personal observation). Environmental conditions were different between 2016 and 2017 in the periods during which inflorescences were developing and cultures were initiated (June through the middle of August). Daytime temperatures in 2017 averaged 3.1 ℃ cooler and rainfall was 125 mm higher than in 2016 (www.weather.net, Griffin-Dempsey Farm, accessed 09 Jul 2018). Cooler, wetter conditions in the field may have been suboptimal for inflorescence development and the ability of the inflorescences to generate callus and shoots when cultured.

Genotypes BX6-7 and Bx8-4 had high scores 30 days after culture initiation in both years, though BX6-9 produced the most rooted plants. Number of rooted plants obtained for each genotype was greater in 2017 (Table 2.4). The use of subculture media with cytokinins was more effective at inducing shoot proliferation, which led to an increase in rooted plants over 2016. However, days to rooting from culture initiation was higher in 2017 (Table 2.4), likely reflecting the amount of time cultures spent in shoot proliferation before rooting.

Conclusion

Our study provides a protocol for the rapid regeneration of little bluestem through micropropagation of immature inflorescences. We were able to produce large numbers of rooted plants that established easily in the greenhouse and grew true-to-type. Our recommendation is to use initiation media consisting of MS salts and vitamins with 1.0 mg•L^{-1} BAP and 0.5 mg•L^{-1} 2,4-D. Cultures should be initiated and subcultured under light. Once callus has formed, cultures should be transferred to subculture medium and subcultured every 30 to 45 days. Subculture medium should consist of MS media containing 1.0 mg•L^{-1} BAP and without 2,4-D. Once shoots have formed, continue to subculture on subculture medium alternating with medium containing no additional growth regulators every 30 to 45 days until roots form. The rooted plants may then be transplanted and acclimated in a greenhouse under intermittent mist. Our regeneration system should prove helpful to growers and researchers.

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Table 2.1. Genotypes of little bluestem used in micropropagation study according to year.

Genotype	**Year**
'Cinnamon Girl'	2016
'Good Vibrations'	2016
'Seasons in the Sun'	2016, 2017
B24-3	2016
BX10-4	2016
BX6-7	2016, 2017
BX6-9	2016, 2017
BX8-4	2016, 2017

Table 2.2. Rating system for scoring of initial cultures of little bluestem after 30 days.

Score	Interpretation
0	Explant died
1	At least some callus formed, but little or no growth occurred
2	Tissue exhibited some callus growth, but no more than 1 leaf or shoot had formed
3	Tissues had doubled in size, and leaves, shoot buds, or shoots were present
4	Tissues had tripled or quadrupled in size and consisted mostly of shoot buds and/or shoots
5	Surface of the medium was covered with shoot buds and/or shoots

Table 2.3. Scores of little bluestem cultures on initiation media rated 30 days after initial culture date, number rooted per explant, and days to rooting.

			2016			2017		
		2, 4-D (mg/L)	Scores 30 days after initial culture	Number rooted per initial culture	Days to rooting from initial culture date	Scores 30 days after initial culture	Number rooted per explant cultured	Days to rooting from initial culture date
Kinetin (mg/L)	1.0	0.5	1.68 ± 0.14 a	6.89 ± 1.23 a	122.01 ± 2.90 a	0.34 ± 0.06 b	14.37 ± 3.09 b	161.01 ± 2.62 b
		1.0	1.43 ± 0.15 a	7.43 ± 1.09 a	109.74 ± 2.68 b	-	-	-
	2.0	0.5	0.81 ± 0.18 b	4.41 ± 1.30 ab	111.67 ± 3.29 b	-	-	-
		1.0	0.93 ±	3.52 ±	110.27 ±	-	-	-

			0.16 b	0.97 b	3.43 b			
BAP (mg/L)	**1.0**	**0.5**	-	-	-	0.96 ± 0.06 a	33.32 ± 3.79 a	199.75 ± 2.45 a

Values represent means ± SE. Means within a column followed by different letters are significantly different at $P < 0.05$. Dash indicates that the treatment combination was not used for the year listed. Only one level of 2,4-D was used in 2017.

Table 2.4. Effect of genotype on scores of little bluestem cultures rated 30 days after initial culture date, number rooted per explant, and days to rooting.

	2016			2017		
Genotype	**Scores 30 days after initial culture**	**Number rooted per culture scored and placed on subculture media**	**Days to rooting from initial culture date**	**Scores 30 days after initial culture**	**Number rooted per culture scored and placed on subculture media**	**Days to rooting from initial culture date**
'Cinnamon Girl'	0.99 ± 0.22 bc	5.19 ± 1.44 b	125.59 ± 4.21 a	-	-	-
'Good Vibrations'	1.68 ± 0.20 a	3.94 ± 1.14 b	105.08 ± 3.05 b	-	-	-
'Seasons in the Sun'	1.36 ± 0.17 ab	4.11 ± 1.14 b	116.62 ± 3.09 a	0.30 ± 0.07 b	16.47 ± 4.08 b	196.6 1± 8.93 a

	0.88 ± 0.33		115.96 ± 4.30			
B24-3[z]	bc	-	a	-	-	-
			106.35 ± 8.62			
BX10-4	0.52 ± 0.28 c	2.84 ± 2.45 b	ab	-	-	-
	1.13 ± 0.24		112.17 ± 4.03			181.36 ± 3.04
BX6-7	abc	3.40 ± 1.70 b	ab	1.05 ± 0.07 a	41.71 ± 6.15 a	a
	0.88 ± 0.31		101.24 ± 4.37	0.31 ± 0.08	48.93 ± 13.88	189.09 ± 5.08
BX6-9	bc	14.09 ± 2.20 a	b	b	a	a
			126.12 ± 3.97		22.60 ± 3.70	186.85 ± 3.54
BX8-4	1.77 ± 0.25 a	5.36 ± 1.14 b	a	0.88 ± 0.07 a	b	a

Values represent means ± SE. Means within a column followed by different letters are significantly different at $P < 0.05$.

[z]Genotype B24-3 was removed from the analysis of number rooted per culture scored and placed on rooting media since rooted plants of this genotype were not fully replicated across each combination of initiation media, initial light treatment, and rooting media.

Table 2.5. Effect of subculture media on number of days to rooting of little bluestem cultures in 2016.

Subculture medium	**Days to rooting**	**Number rooted**
Without NAA	110.31 ± 1.89 b	2.22 ± 0.31
0.1 mg•L^{-1} NAA	122.66 ± 3.08 a	1.55 ± 0.26

Values represent means ± SE. Means within a column followed by different letters are significantly different at $P < 0.05$. Number rooted by subculture media calculated over all genotypes.

Figure 2.1. Immature inflorescence of little bluestem with ruler for scale reference. Inflorescence were cut into pieces ≈ 5-10 mm long to produce explants for culturing.

Figure 2.2. Little bluestem cultures several weeks after scoring and during shoot and root formation.

Figure 2.3. Little bluestem plants produced through micropropagation.

CHAPTER 3

Little Bluestem (*Schizachyrium scoparium* L.) Diversity in Three Regions –the Midwest, New England, and Georgia[2]

[2] Hawkins, S.M., K. Harris-Shultz, M. Harrison, and C. Robacker. To be submitted to *HortScience*.

Abstract

Little bluestem (*Schizachyrium scoparium* L.) has increased in popularity as an ornamental grass. Genetic and phenotypic variability in little bluestem makes it a good candidate for breeding new cultivars. Our objective was to assess genetic diversity among little bluestem genotypes from three regions in the United States: the Midwest, New England, and the Southeast, as represented by Georgia. We assessed genetic diversity of 49 genotypes of little bluestem using 10 polymorphic simple sequence repeat markers (SSRs) that had been developed for the little bluestem cultivar 'The Blues'. An AMOVA revealed that 13.1% of the variation was among the three regions and 86.9% of the variation was within the regions. Nei's genetic distance was highest between the Midwest group and the Georgia group, and smallest between New England and the Midwest group. Cluster analysis in Structure showed three groups for the entire sample set. However, genotypes did not cluster together by region. Hierarchical structure analysis in Structure for genotypes from New England and for the Midwest revealed three groups for the Midwest and two groups for New England. Gene flow between regions is likely influenced by pollen dispersal on prevailing westerly winds, ecological similarities between New England and Georgia, and possible post-glacial migrations between Georgia and New England. Further germplasm collections in the Southeast could allow for genetic diversity studies and clarification of the genetic relationship of little bluestem from New England and the southeastern United States, as well as providing new germplasm for the development of cultivars with good regional adaptation.

Little bluestem (*Schizachyrium scoparium* L.) is a long-lived perennial bunchgrass native to most of the continental United States, as well as northern Mexico and southern Canada (Fu et al., 2004; Springer, 2012; Williams and Briske, 1991). Little bluestem is a tetraploid (2n = 4x = 40) and is considered to be a segmental allotetraploid (Church, 1940; Dewald and Jalal, 1974; Springer, 2012). Little bluestem exhibits adaptation to a variety of soils and climates and has high phenotypic plasticity (Boe and Bortnem, 2009; Springer, 2012). Although earliest breeding efforts in little bluestem were to develop forage varieties, in recent years breeding of ornamental little bluestem varieties has increased in response to the increasing popularity of ornamental grasses in the landscape (Anderson and Aldous, 1938; Fu et al., 2004; Meyer, 2012; Meyer et al., 2017). Little bluestem has many desirable ornamental traits including good fall color, a variety of growth habits, and attractive seed heads that persist through the winter (Boe and Bortnem, 2009; Cullina, 2007; Springer, 2012).

A high amount of genetic variability has been discovered in little bluestem. A genetic diversity study using RAPD markers with populations of little bluestem from Oklahoma and New Jersey showed that 95% of genetic variation was within populations (Huff et al., 1998). A study using AFLP markers revealed that 91.5% to 92.8% of the genetic variation of six populations of little bluestem from Manitoba, Canada was within populations although significant variance also existed among populations (Fu et al., 2004). Interpopulation distance between pairs of little bluestem populations increased slightly with geographic distance (Fu et al., 2004). Genetic diversity was assessed among accessions from the USDA-Agricultural Research Service (ARS) National Plant Germplasm System (NPGS) collection using microsatellite markers developed for little bluestem using the cultivar 'The Blues' (Harris-Shultz et al., 2015). Most of the USDA accessions evaluated were from the Midwestern region of the

United States, although an accession from Rhode Island was also included in the study (Harris-Shultz et al., 2015). Contrary to previous studies, little genetic diversity was found among accessions (Harris-Shultz et al., 2015).

To investigate the genetic diversity of little bluestem genotypes from the Midwest, New England, and Georgia, we used SSR markers developed by Harris-Shultz et al. (2015). Our objective was to determine the genetic variation within and among the sample sets from each region.

Materials and Methods

Plant Material

Genotypes from three regions were used in the study (Table 3.1). Three of the genotypes were cultivars from the joint breeding program of Robacker (University of Georgia) and Harrison (USDA). The selections from the UGA-USDA breeding program were 'Cinnamon Girl', 'Seasons in the Sun', and 'Good Vibrations'. 'Blue Heaven' was used as a control to check amplification of primers since it had been used in a previous study (Harris-Shultz et al., 2015). Young leaves were collected from the cultivars in the UGA-USDA breeding program, as well as from 'Blue Heaven', in Robacker's field plots in Griffin, GA. Seeds of additional genotypes were collected from locations within Georgia. The remainder of the genotypes were supplied by the USDA-ARS NPGS as accessions. Each accession, as represented by a PI number, consists of a population of multiple genotypes. USDA-provided genotypes and Georgia-collected genotypes were grown from seed, and leaf material was collected from young seedlings.

DNA Extraction and PCR Amplification

Total genomic DNA from 76 genotypes of little bluestem was extracted using a modified CTAB method (Doyle and Doyle, 1987). Concentration of DNA in samples was determined using a spectrophotometer (NanoDrop 2000, ThermoFisher Scientific, Waltham, MA).

DNA samples were aliquoted into a 96-well plate for the diversity study. Two samples of 'Blue Heaven' were included in each plate to verify amplification. SSR primers from a previous study by Harris-Shultz et al. (2015) were used (Table 3.2). DNA was amplified using a 10-µl reaction volume containing 2.0 µl of 5x Green GoTaq® Flexi reaction buffer (Promega, Madison WI), 1.0 µl of 25 mM $MgCl_2$, 0.80 µl of 2.5 mM dNTP mix, 1.8 µl of 1.0 µM M13 primer (Integrated DNA Technologies, Coralville, IA) labelled with IR-Dye 700 (Integrated Data Technologies, Coralville, IA), 0.04 µl Go Taq® Polymerase (Promega, Madison, WI), 0.86 µl sterile water, 0.5 µl of 1.0 µM M-13 labelled forward primer, 2.0 µl of 1.0 µM reverse primer, and 1.0 µl of 2.5 ng•μl^{-1} DNA. Thirty-five primers were amplified (Table 3.2). Thermocycler conditions were: 3 minutes at 94 ºC, 39 cycles at 94 ºC for 30 seconds, 45 to 60 ºC for 1 minute, 72 ºC for 1 minute 10 seconds, and 10 minutes at 72 ºC (Harris-Shultz et al., 2015). For primers that did not amplify during testing using the original protocol, a touchdown program (Hao et al., 2008) was used and thermocycler conditions were: 3 minutes at 95 ºC; 10 cycles of 94 ºC for 40 seconds, 65 ºC for 45 seconds (-1 ºC per cycle) and 72 ºC for 1 minute; 32 cycles of 94.0 ºC for 40 seconds, 55 ºC for 45 seconds, and 72 ºC for 1 minute; and 72 ºC for 10 minutes. A Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA) was used for amplification. 9.0 µl of the individual PCR products was loaded into a 6.0% w/v polyacrylamide gel using a Mega-Gel High Throughput Vertical System (C.B.S. Scientific, Del Ma, CA) (Wang et al., 2003).

Bands were visually scored and assigned a score of "1" for a present band and "0" for an absent band. Missing bands were scored as 9 for NTSYSpc and -1 for Structure.

Individual samples that had more than 25% missing data were eliminated from the analysis. Monomorphic and poorly amplifying markers were also removed. The elimination of individual samples with more than 25% missing data caused three polymorphic primers (147859, 148046, and 148597) to become monomorphic and the primers were removed from the analysis, leaving 10 polymorphic markers. Two samples of 'Blue Heaven', the genotype used for a control, were kept. Removal of monomorphic and poorly amplifying markers, and individual samples with missing data (Table 3.2) left 50 samples of 49 different genotypes (Table 3.1) to be analyzed. Of the genotypes analyzed in the study, 21 were from New England, 19 were from the Midwest, and 9 were from Georgia. Removal of individual samples with a high percentage of missing data resulted in the complete elimination of samples from three of the five collection sites in Georgia.

Data Analysis

Marker data was imported into NTSYSpc version 2.2 (Rolf, 2008) for unweighted pair group method with arithmetic mean (UPGMA) cluster analysis. The SIMQUAL model for genetic similarity with the DICE coefficient (Nei and Li, 1979) was used to calculate genetic similarity. A dendrogram was generated using the UPGMA procedure in the SAHN module. Bootstrapping analysis was done in FreeTree (Hampl et al., 2001) with 1000 repetitions. Bootstrap values are only shown if 50% or higher. Bayesian cluster analysis was performed using Structure Version 2.3.4 (Pritchard et al., 2000). Putative population data was assigned to each sample in the data file for Structure, with individual samples assigned to each region (Midwest, New England, and Georgia). A burn-in period of 50,000 and 50,000 Markov Chain

Monte Carlo (MCMC) repetitions were used. The parameters were set to an admixture model with allele frequencies correlated. The L(K) procedure (Pritchard et al., 2000) was used by running a batch job for K = 1 to K = 10 to determine the best number of populations. The delta K procedure (Evanno et al., 2005) was used by running a batch job for K = 1 to K = 10 for 20 iterations. The program Structure Harvester was used to calculate ΔK (Earl and Vonholdt, 2012). Separate batch jobs were run in Structure for the entire sample set to include and exclude prior population information for comparison.

After the initial procedures for the dataset that included samples from all populations, a hierarchical Structure analysis was performed for the Midwest and New England subpopulations. The procedures were repeated in NTSYSpc, FreeTree, Structure, and Structure Harvester for the datasets containing only the Midwest or New England subpopulations. The same Structure parameters were used when running batch jobs for the Midwest subpopulation as for the total population. However, for the New England subpopulation, the Structure parameters were modified to use a burn-in period of 200,000 and 200,000 MCMC repetitions. Dendrograms were generated for each subpopulation.

A binary matrix of presence/absence data for each allele (Assoumane et al., 2013; Teixeira et al., 2014; Zhang et al., 2011) was used for analysis in GenAlEx version 6.5 (Peakall and Smouse, 2012). Number of alleles, Shannon's information index, and genetic diversity were calculated for each population. An analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was also performed in GenAlEx. The AMOVA was calculated for all samples partitioned by region of collection and was run with 9,999 permutations. Nei's Distance among populations and population genetic variation (PhiPT) calculations were performed in GenAlEx (Assoumane et al., 2013; Teixeira et al., 2014; Yamasaki and Ideta, 2013). PhiPT, an equivalent measure to

F_{ST}, calculates differences among population while allowing within population variation to be suppressed (Teixeira et al., 2014; Yamasaki and Ideta, 2013).

Results and Discussion

Genetic Diversity

A total of 33 alleles were amplified from the 10 polymorphic microsatellite loci. Among regions, number of alleles (N_A) ranged from 1.0 to 1.5 per loci and fragment size combination and number of effective alleles (N_E) among populations ranged from 1.1 to 1.2 (Table 3.3). Shannon's Information Index (I) ranged from 0.15 to 0.23 among regions (Table 3.3). Expected heterozygosity (H_E) ranged from 0.09 in Georgia to 0.14 in the Midwest, while unbiased expected heterozygosity (uH_E) ranged from 0.10 to 0.15 for Georgia and the Midwest respectively (Table 3.3). The Midwest and New England groups had two private alleles each. The Georgia group had three private alleles, a higher number than the other two groups even though the sample size for Georgia was smaller. The higher number of private alleles in the Georgia samples could indicate reduced gene flow between Georgia and the other two groups.

All variance components of the AMOVA were significant ($p = 0.0001$) (Table 3.4). Differences among populations explained only 13.1% of the variation, while differences within populations accounted for 86.9%. The low variation among populations is characteristic of an outcrossing species with wide-spread distribution and wind-dispersed seeds (Hamrick and Godt, 1996).

Our results are similar to previous studies of genetic diversity in little bluestem (Fu et al., 2004; Huff et al., 1998). Fu et al. (2004) showed a variation of 7.2% among populations and 92.8% within populations from tiller samples. Seed samples had a variation of 8.5% among populations and 91.5% within populations (Fu et al., 2004). Huff et al. (1998) showed a variation

of 5% among populations and 95% within individuals within populations. Although the variation among populations is higher in our study than in the two previous studies, all three studies show a pattern of small among population variation and large variation among individuals within a population.

Nei's distance was greatest between the Midwest group and the Georgia group and was least between the Midwest group and the New England group (Table 3.5). However, all distances were small and ranged from 0.032 and 0.089. Pairwise genetic variation between regions as measured by PhiPT was highest between the Midwest group and the Georgia group at 0.21. PhiPT was almost identical between the New England group and the Georgia group and between the New England group and the Midwest group at 0.10 and 0.11 respectively (Table 3.5).

Genetic distance and population differentiation as measured by Nei's distance and PhiPT was highest between the Midwest group and the Georgia group. Higher genetic distances likely reflect a larger geographic distance between the two regions than between New England and either of the regions. Genetic distance was correlated with geographic distance between populations of little bluestem within the Canadian province of Canada (Fu et al., 2004) and would likely increase with longer distances between populations.

Genetic distance between the Midwest group and the New England group was less than genetic distance between the New England group and the Georgia group. New England collection sites are far from the Georgia collection sites and from many of the Midwestern sites such as those in New Mexico and Wyoming. However, genotypes from collection sites in Ohio were also included in the Midwest group and no doubt contributed to the smaller distance between the New England and Midwest groups as the Ohio sites were closer to New England than any other collection sites in the Midwest or any sites in the Georgia group. Prevailing

westerly winds could have contributed to gene flow through pollen dispersal (Casler et al., 2007; Zhang et al., 2011) between Ohio and New England and contributed to the smaller genetic distance between the Midwest and New England groups.

Population Structure

UPGMA cluster analysis in NTSYSpc for all little bluestem samples showed three major groups with smaller sub-groups, although most sub-groups were not well-supported by bootstrapping (Figure 3.1). However, groups did not segregate by region. Group 1 contained samples from all three regions. In Group 1, 19 of the 21 New England samples clustered, as well as 5 of the 9 samples from Georgia. Group 1 also included all the samples from the Midwest region except for 'Cinnamon Girl'. Group 2 included the remaining four samples from Georgia and one sample from New England. Group 3 included only 'Cinnamon Girl' and one sample of PI 677213 from New England.

Cluster analysis in Structure of all samples and not using prior population information revealed that K = 3 (Figure 3.2). However, membership in groups was different between results from UPGMA analysis and those in Structure. In Structure, Group 1 and 2 largely corresponded to Group 1 in the UPGMA dendrogram. Group 1 in Structure included 'Good Vibrations' as well as 3 additional samples from the Midwest group, 16 of the 21 New England samples, and 1 sample from Georgia (Figure 3.2). Group 2 in Structure included 9 samples from the Midwest group, 4 samples from New England, and the remaining 8 samples from Georgia. Group 3 in Structure included the three samples of PI 216751 from Texas, 'Seasons in the Sun', 'Cinnamon Girl', both samples of 'Blue Heaven', and one sample of PI 677213 from New England. The female parents of the UGA/USDA cultivars were genotypes from Texas. Cultivars were the results of open-pollinated crosses in crossing blocks which included accessions from other

regions, including five plants from PI 213875 from Rhode Island. The male parent of 'Cinnamon Girl' might have originated in New England, causing the cultivar to cluster with the New England accession.

Bayesian cluster analysis in Structure of all samples using prior population information also gave a result of K = 3 (Figure 3.3). Membership in groups differed between results from UPGMA analysis. However, membership in groups for the Bayesian analysis using prior population location was the same as in the analysis without prior population information.

UPGMA cluster analysis in NTSYSpc for the Midwest set of little bluestem samples showed three major groups (Figure 3.4). Most smaller groups were not well supported by bootstrapping. Group 1 included 16 of the 20 samples, including 'Good Vibrations', 'Seasons in the Sun', and 'Blue Heaven'. Group 2 included two samples of PI 648373 from Kansas and one sample of PI 476298 from Wyoming. Group 3 consisted of only 'Cinnamon Girl'.

Bayesian cluster analysis in Structure for the Midwest set of samples revealed that K = 3 (Figure 3.5). Membership in groups was again different between the UPGMA analysis and the analysis in Structure. In Structure, Group 1 and 2 corresponded to Group 1 in NYSYSpc. Structure Group 3 corresponded to NTSYSpc groups 2 and 3. The only other difference between the groupings was that 'Cinnamon Girl' clustered with 'Seasons in the Sun', 'Good Vibrations', and 'Blue Heaven', as well as the three samples of PI 216751 from Texas in Structure Group 2.

UPGMA cluster analysis in NTSYSpc for New England little bluestem showed two major groups (Figure 3.6), although the groups were not well-supported by bootstrapping. The only bootstrap value over 50% was that for the main two groups. Group I included only four samples: one sample of PI 677206 and three samples of PI 677213. Group II included all other New England samples.

Initial cluster analysis in Structure for New England little bluestem showed that K=2, although several smaller peaks were seen in Structure Harvester. Clusters analysis from running Structure nine times with a burn-in period of 200,000 and 200,000 MCMC reps showed K=2 for seven out of nine runs and K=3 for the remaining two runs. Therefore, we selected K=2 as the most accurate number of populations (Figure 3.7). Group 1 in Structure corresponded to Group 2 in the UPGMA analysis and Group 2 corresponded to Group 1 in the UPGMA analysis. In Structure, Group 1 had 14 samples and Group 2 had 7 samples. Group 2 included the four samples that comprised Group 1 in the UPGMA analysis. However, Group 2 in Structure also included one sample of PI 677190, an additional sample of PI 677213, and one sample of PI 677193. Group 1 in Structure included all other samples. The three additional samples in Group 2 of Structure were the only differences in group membership between Structure and UPGMA analysis in NTSYSpc.

The lack of clearly defined population structure within and among regions is not surprising. Gene flow is influenced by many factors, including mode of reproduction, seed dispersal mechanisms, and geographic proximity of populations (Hamrick and Godt, 1996). Little bluestem, as an outcrossing species that uses wind to disperse both pollen and seeds, is similar to other members of the Poaceae family in having high gene flow and low population structure. Genotypes of species and hybrids of the tropical forage grass *Urochloa* P. Beauv. collected in Tanzania had low population differentiation and high gene flow (Kuwi et al., 2018). An AMOVA of the *Urochloa* grasses showed that 92% of the variance was within individuals and only 5% was among populations (Kuwi et al., 2018). An AMOVA from a genetic diversity study of big bluestem (*Andropogon gerardii* Vitman) from Wisconsin and the Northeast revealed that 4% of the variance was among groups and 86% of the variance was within plants within

populations (Price et al., 2012). However, a genetic diversity study of big bluestem from Kansas and Illinois showed a 12.5% variance among populations and 87.5% within populations (Gustafson et al., 2004). Results were similar for Indiangrass [*Sorghastrum nutans* (L.) Nash] from Kansas and Illinois where among population variance was 12.2% and within population variance was 87.8% (Gustafson et al., 2004).

Gene flow between regions may be influenced by different factors. Gene flow between the Midwest and New England could occur in part due to pollen dispersal on prevailing westerly winds (Casler et al., 2007; Zhang et al., 2011). Post-glacial migrations may influence gene flow between Georgia and New England. Switchgrass (*Panicum virgatum* L.) cultivars Alamo and Kanlow, developed in the Great Plains, as well as other accessions from the Midwest, showed evidence of descending from genotypes originating from a glacial refuge on the western part of the Gulf Coast (Zhang et al., 2011). Lowland accessions of switchgrass in the eastern United States showed evidence of descending from genotypes in a glacial refuge on the eastern Gulf Coast (Zhang et al., 2011). Grasslands, including bluestem prairies, were much more prevalent in the southeastern United States after the last glacial maximum than they currently are (Lamoreaux et al., 2009; Watts, 1971). Possibly a similar process of migration occurred in little bluestem between the Southeastern United States and New England after the last glacial maximum.

Ecological similarities between Georgia and New England could also help explain the greater similarity of samples from Georgia to those from New England than to samples of the Midwest group. Although climactic differences exist between Georgia and New England, both experience far greater rain fall and humidity than the Midwest. Origin in different ecoregions explained a small but significant part of the variance among big bluestem from different ecoregions in the Northeastern United States (Price et al., 2012). Likewise, difference in habitat

was responsible for a small but significant variance among genotypes of the central European grass *Sesleria albicans* Kit. ex Shultes (Reisch et al., 2003). Further investigation of little bluestem genotypes from New England and the southeastern United States might clarify the relationship of little bluestem from both regions and provide new germplasm for breeding and restoration efforts.

To this end, collections of little bluestem across the southeastern United States should be expanded. Although we eliminated little bluestem samples originating from three Georgia collection sites due to an excess of missing data, most samples we eliminated did amplify for some of the polymorphic markers as well as some of the monomorphic markers. The eliminated samples might have been sub-species of little bluestem as some showed phenotypic differences, such as pubescence, from little bluestem germplasm from other collection sites. Little bluestem collected from other sites in the southeastern United States might yield better results in a study of genetic diversity and provide germplasm for regionally adapted cultivars. Additionally, development of additional SSR markers might enable future genetic diversity studies to provide more information on little bluestem from the southeastern United States.

Conclusion

Most of the genetic variation was within regional groups in our study of the genetic diversity of little bluestem from the Midwest, New England, and Georgia. Little bluestem's high heterozygosity and genetic variability, along with its phenotypic variability and adaptability to a wide range of ecological niches, make it a good candidate for continued development of ornamental cultivars. Collection and genetic characterization of new genotypes of little bluestem in the southeastern United States could allow plant breeders to produce cultivars with good regional adaptation.

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Table 3.1. *Schizachyrium scoparium* genotypes examined for genetic variation.

Sample no. on dendrogram	Genotype (Rep)	Original collection location of genotype (or parent material of cultivar)
2	PI 648370 (2)	New Mexico
3	PI 648370 (3)	New Mexico
5	PI 648370 (5)	New Mexico
6	PI 648373 (1)	Kansas
7	PI 648373 (2)	Kansas
9	PI 648373 (4)	Kansas
12	PI 668128 (2)	Ohio
13	PI 668128 (3)	Ohio
17	PI 216751 (2)	Texas
18	PI 216751 (3)	Texas
20	PI 216751 (5)	Texas
22	PI 476298 (2)	Wyoming
23	PI 476298 (3)	Wyoming
24	PI 476298 (4)	Wyoming
25	PI 476298 (5)	Wyoming
26	PI677206 (1)	Maine
28	PI677206 (3)	Maine
29	PI677206 (4)	Maine
30	PI677206 (5)	Maine

31	PI 677190 (1)	Massachusetts
34	PI 677190 (4)	Massachusetts
35	PI 677190 (5)	Massachusetts
36	PI 677213 (1)	Massachusetts
37	PI 677213 (2)	Massachusetts
38	PI 677213 (3)	Massachusetts
39	PI 677213 (4)	Massachusetts
40	PI 677213 (5)	Massachusetts
41	PI 677218 (1)	Rhode Island
42	PI 677218 (2)	Rhode Island
43	PI 677218 (3)	Rhode Island
44	PI 677218 (4)	Rhode Island
46	PI 677193 (1)	Vermont
47	PI 677193 (2)	Vermont
48	PI 677193 (3)	Vermont
49	PI 677193 (4)	Vermont
50	PI 677193 (5)	Vermont
52	‘Good Vibrations’ (2)	UGA/USDA cultivar – Texas
60	‘Seasons in the Sun’ (5)	UGA/USDA cultivar – Texas
64	‘Cinnamon Girl’ (4)	UGA/USDA cultivar – Texas
73	PI 674715 (1)	Georgia
74	PI 674715 (2)	Georgia
75	PI 674715 (3)	Georgia

76	PI 674715 (4)	Georgia
77	PI 674715 (5)	Georgia
84	S. Rockdale Park (2)	Georgia
85	S. Rockdale Park (3)	Georgia
86	S. Rockdale Park (4)	Georgia
87	S. Rockdale Park (5)	Georgia
89, 90	‘Blue Heaven’ (1 and 2)	Minnesota

Table 3.2. Primers used in study, with forward and reverse sequence, PCR conditions, and results of amplification on gel.

Primer	**Forward sequence (5' to 3')**	**Reverse sequence (5' to 3')**	**PCR protocol**	**Annealing temperature in ℃ (for amplification under original conditions)**	**Results of amplification on gel**
7048	GAGTACGAGCTCAACCCAACA	GGATCTTCCATCTTGGCTACC	Original	50	Polymorphic
42074	ACTATGCATCAGGCATTCAGG	CGGGGAATACCATTCTTTGTT	Touchdown	-	Polymorphic
54101	AACTTGGACACGGATCAAGG	TGGTCCAAAGCTCAGTCAGTT	Touchdown	-	Did not amplify
55435	AGCAGAGCTCCATGGTTGAC	CCCACCTCTGCAATTATTCAA	Original	50	Polymorphic
69198	ATATCTCGCAAACAGCT	TTGCAAACCATTGA	Original	50	Too faint to score

	GCAA	CCTTTTC			
74487	GCAAAACTACAAGCAC ATTCAGA	GCATAAGGTATAG GCAGCACAA	Original	50	Monomorphic
146020	CCAAAAGACTACATCA ACATCCA	TTTGGCAGAAAAGC AACTTTC	Touchdown	-	Did not amplify
146104	GCTTCTCTGTTCGTCAC TTGAG	TCATGATCCATCAA CGCTAGA	Original	50	Too faint to score
146227	TACAGAGTGGGGGTAG AGAGC	ATATTTATGATTCG GCCATGC	Touchdown	-	Too faint to score
146802	GATCATTTGGATGTGGA AGGA	TGCATATGTTCTCG TTGACCA	Original	50	Too faint to score
147498	GAGGCGGGCTCTTTACA TTT	TGTCACTTTGTTTA TGCGTCTTT	Original	45	Did not amplify
147679	TCTCTCAACTGATGCTT GCTC	TGACGTTCATCAAC TCAGGACT	Original	45	Polymorphic
147859	CCCAAGAGCTCAACTTG	AGGCTTCTCTACGC	Original	45	Polymorphic, but

	CTTA	CAGAAAC			removed from analysis
147951	TAGGTGTGTGGAGAACC CTCA	TCATAGACCCGCAA ATTCATC	Touchdown	-	Too faint to score
148046	GTTTCCATTTTCAGGCC ATTT	GACCTCCCTTCTCC TCCTTCT	Original	60	Polymorphic, but removed from analysis
148066	CCGGAGAGAGAGAGAG AGGAG	GCCGTCAGTACGGG AAGTT	Original	45	Too faint to score
148597	TGTGAGTGTAGGACCAA CGTG	CACACAAACATTAC GCAGCAT	Original	50	Polymorphic, but removed from analysis
148706	GGATGGTATAATGTTTC CCATTT	TTCCTTCAATCCTTT TCTTTGC	Touchdown	-	Too faint to score
148812	AGATGCTTCATCGGAAA TTCA	CAAAGCAGCAGCA AAAAGAAC	Touchdown	-	Did not amplify

148974	AAAAACCGCCACCATTATGTA	TTTGACCTTTTTATGACTAATGC	Original	50	Too faint to score
149014	TGGGCACACCGTCTATTGTAT	CCTTTGGTCTTGTGCTCTTGT	Original	50	Polymorphic
149427	TTCCGTGGCAGTAGGACAATA	TGCTGCTCTCTTTTGGATTTG	Original	50	Monomorphic
151116	GTTTTTCGTGGCAGTAGGACA	TGCTGCTCTCTTTTGGATTTG	Original	60	Too faint to score
153072	TGGCTGTTGTGGTTCTTTACC	CGCTTGGAGACTAGCAATCAA	Touchdown	-	Polymorphic
153354	CGGACTCCTACACACGTAAGC	AGTACCTGATCTTCGGGCTGT	Touchdown	-	Monomorphic
160531	GGGCAAACTTGGAAGAGACTT	GACGTTCACTTTATCCGAGCA	Original	50	Did not amplify
161460	ACCATCAAATGTGCTTGGAGT	GAGCATTGAGTTGAGGATTGC	Original	45	Polymorphic

161551	TCACCTCTCCTTCAATT CATTTC	ATGGTGTCATGCTT CCATTCT	Touchdown	-	Did not amplify
166558	GACACCTCCTCCAGTTC CTTC	AACCCAAGCTTAGG AGTCACC	Original	50	Polymorphic
174261	CCAGTGTCGAAGTTGGA TCAT	CATATCGTGTCGGC TATCCTC	Original	50	Monomorphic
HE586094	TCCCTTTCTTTCCTGGGT TT	AAAAATTTCCACGG GTTCG	Touchdown	-	Polymorphic
JQ951633	GACAGGCACAGCAAGA TCC	CTTGCTAGCCTTGT CGAAGC	Original	50	Polymorphic
JQ951654	ACATGTCACACCGTGTT GCT	GCCCAGCTTTTGTA ATGGAA	Original	50	Monomorphic
JQ951715	GCTCCTCCAAGGACAAG ATG	GGGATTGTAGTGCA CGGTGT	Touchdown	-	Did not amplify
JQ951737	CTGATGCCGGAGACAA GAA	AGTGCACAACTGA GCACCTTT	Original	50	Too faint to score

Table 3.3. Summary statistics for *Schizachyrium scoparium* samples from three regions – Midwest, New England, Georgia.

Region	**N**	**Na**	**Ne**	**I**	**He**	**uHe**
Midwest	20	1.5	1.2	0.23	0.14	0.15
New England	21	1.5	1.2	0.20	0.13	0.13
Georgia	9	1.0	1.1	0.15	0.09	0.10
Total	50	1.3	1.2	0.19	0.12	0.12

N – number of samples, N_A - number of alleles, N_E - number of effective alleles, I - Shannon's Information Index, H_E - expected heterozygosity, uH_E - unbiased expected heterozygosity

Table 3.4. AMOVA for regional groups of *Schizachyrium scoparium*.

Source	***d.f.***	**SS**	**Est. var.**	**%**
Among populations	2	32.928	0.734	13.1%
Within populations	47	229.212	4.877	86.9%
Total	49	262.140	5.611	100.0%

Source – source of genetic variation, d.f. – degrees of freedom, SS – sum of squares, Est. var. – estimated variance, % - percent of genetic variation explained by source of variation

Table 3.5. Pairwise Nei's distance values (below the diagonal) and PhiPt (above the diagonal) for regional groups of *Schizachyrium scoparium*.

	Midwest	**New England**	**Georgia**
Midwest	0.000	0.106	0.215
New England	0.032	0.000	0.099
Georgia	0.089	0.059	0.000

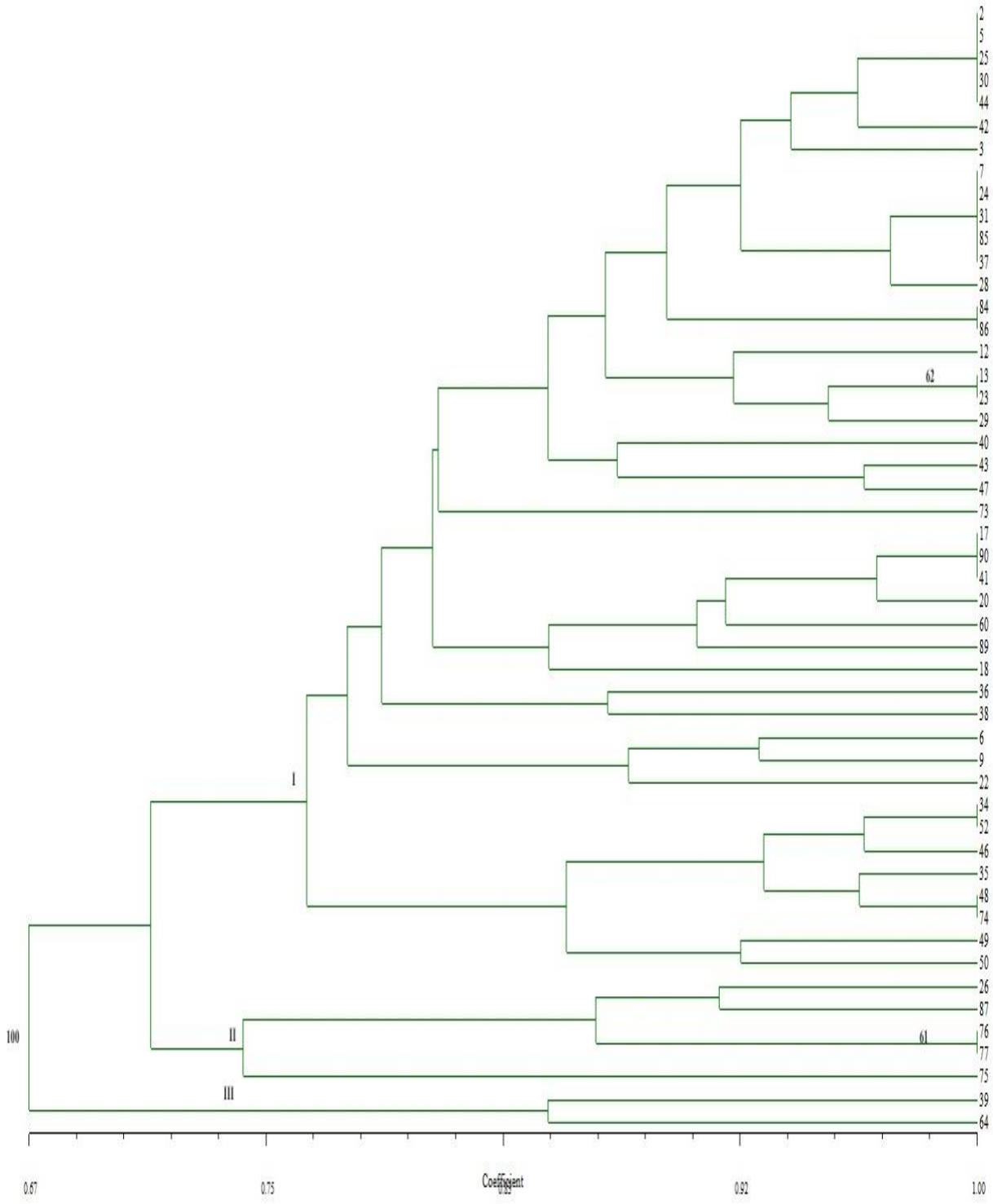

Figure 3.1. UPGMA cluster analysis for *Schizachyrium scoparium* samples from all regions – Midwest, New England, and Georgi

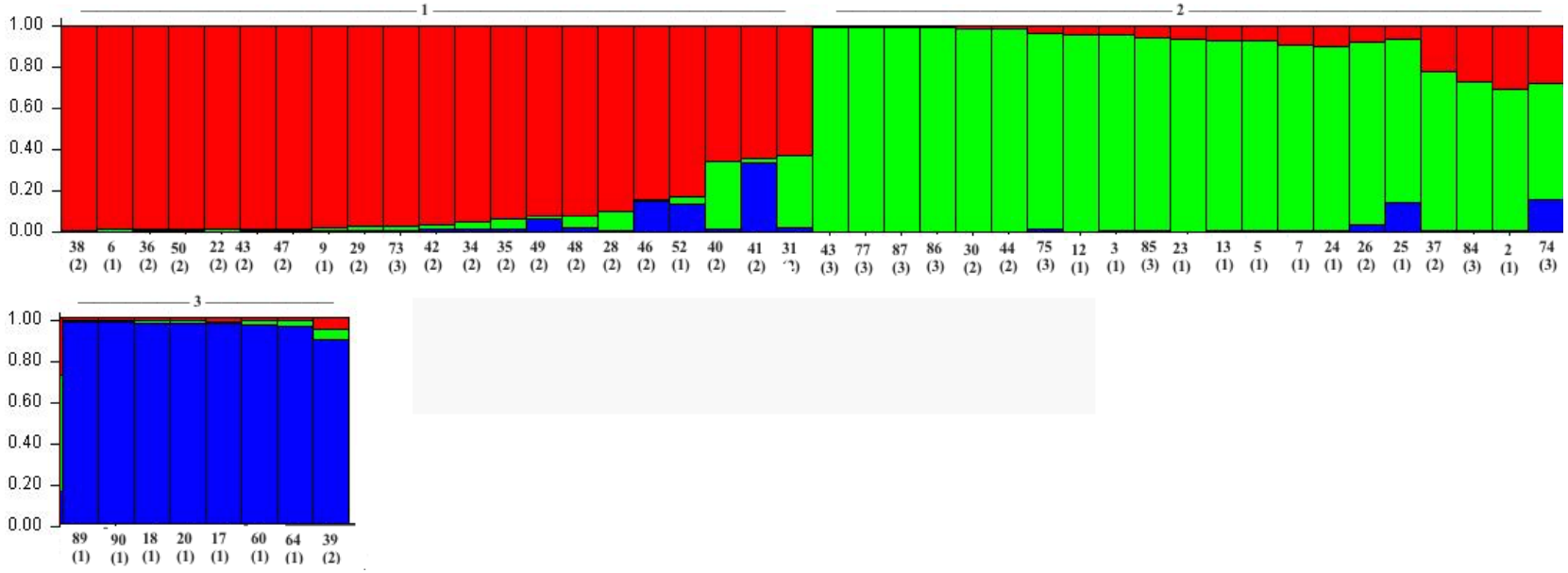

Figure 3.2. Bayesian cluster analysis for all *Schizachyrium scoparium* samples without using prior population information. Numbers on labels correspond to sample number on dendrogram. Numbers in parentheses are the number of the assigned population from collection regions. 1 –Midwest, 2 – New England, 3 – Georgia.

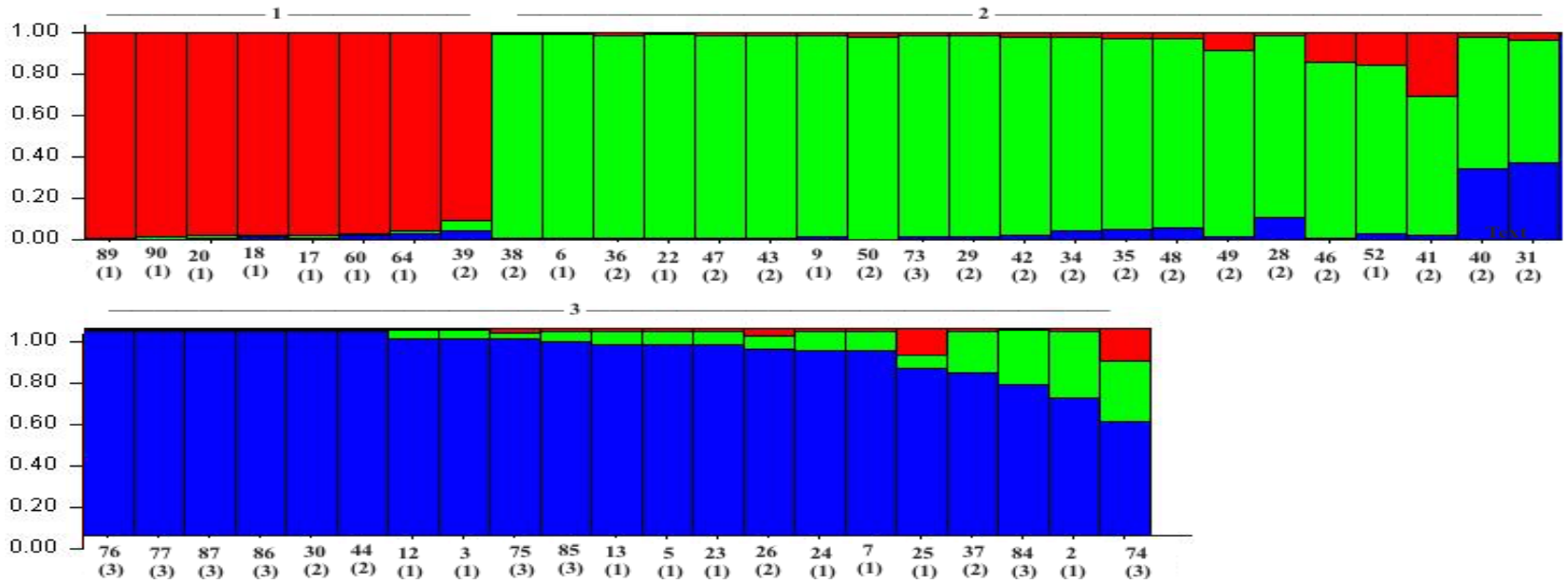

Figure 3.3. Bayesian cluster analysis for all *Schizachyrium scoparium* samples using prior population data. Numbers on labels correspond to sample number on dendrogram. Numbers in parentheses are the number of the assigned population from collection regions. 1 –Midwest, 2 – New England, 3 – Georgia.

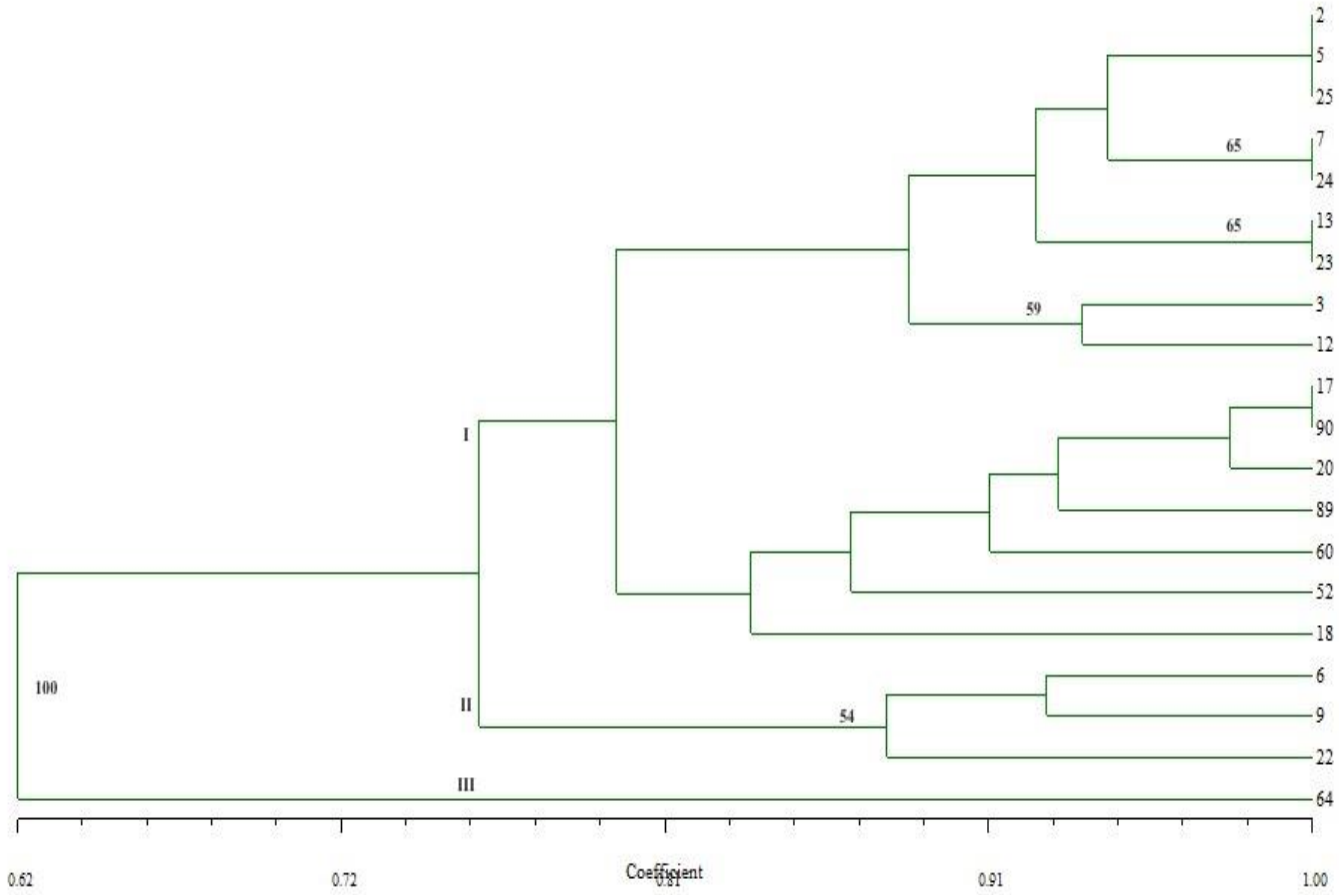

Figure 3.4. UPGMA cluster analysis for *Schizachyrium scoparium* samples from the Midwest.

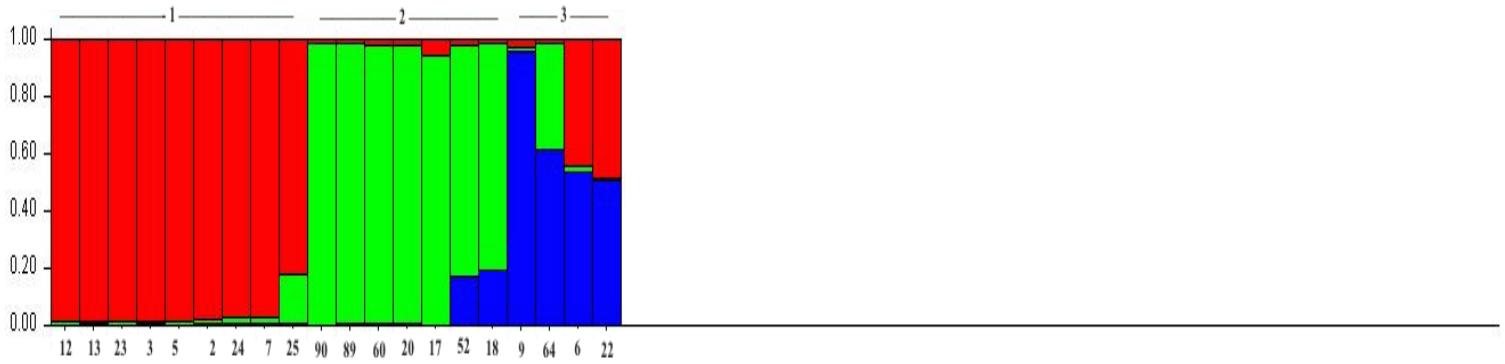

Figure 3.5. Bayesian cluster analysis for *Schizachyrium scoparium* samples from the Midwest. Numbers on labels correspond to sample number on dendrogram.

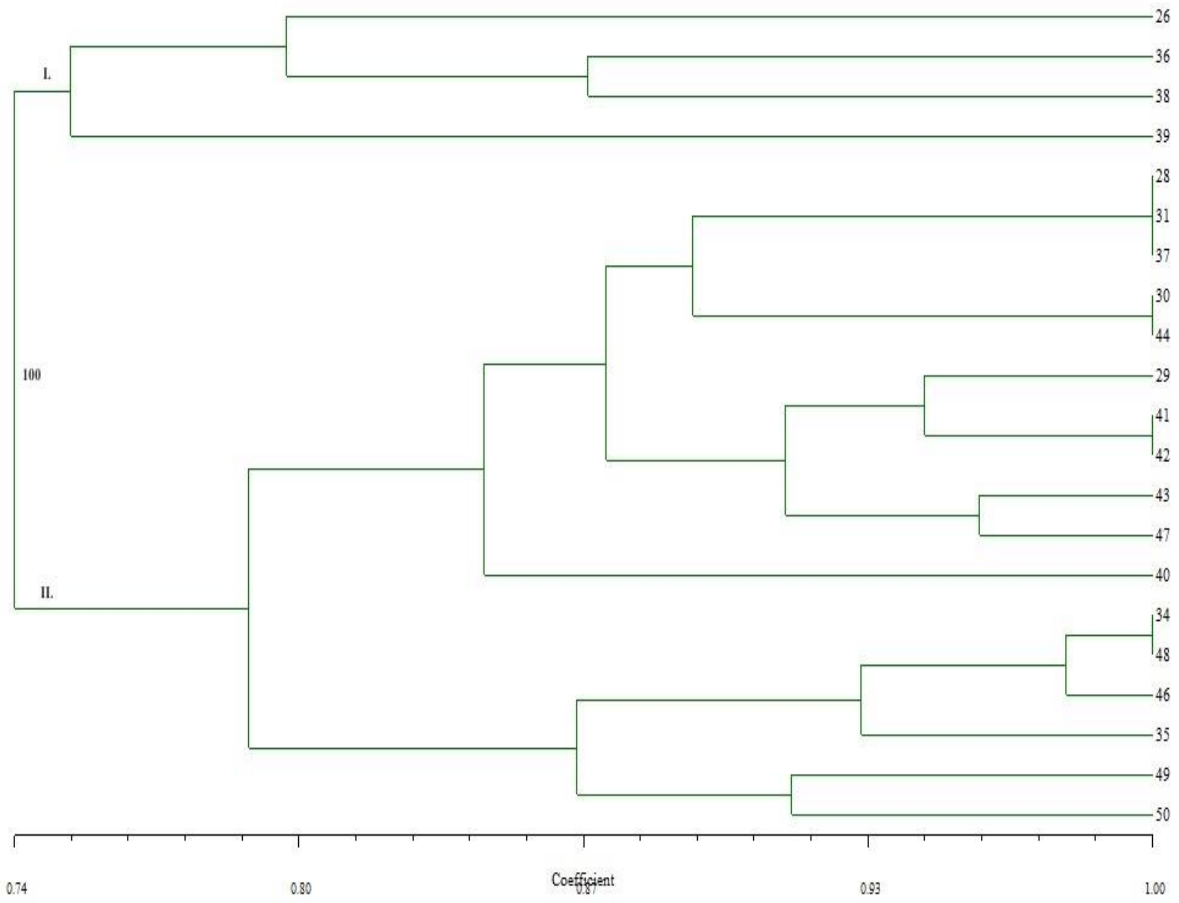

Figure 3.6. UPGMA cluster analysis for *Schizachyrium scoparium* samples from New England.

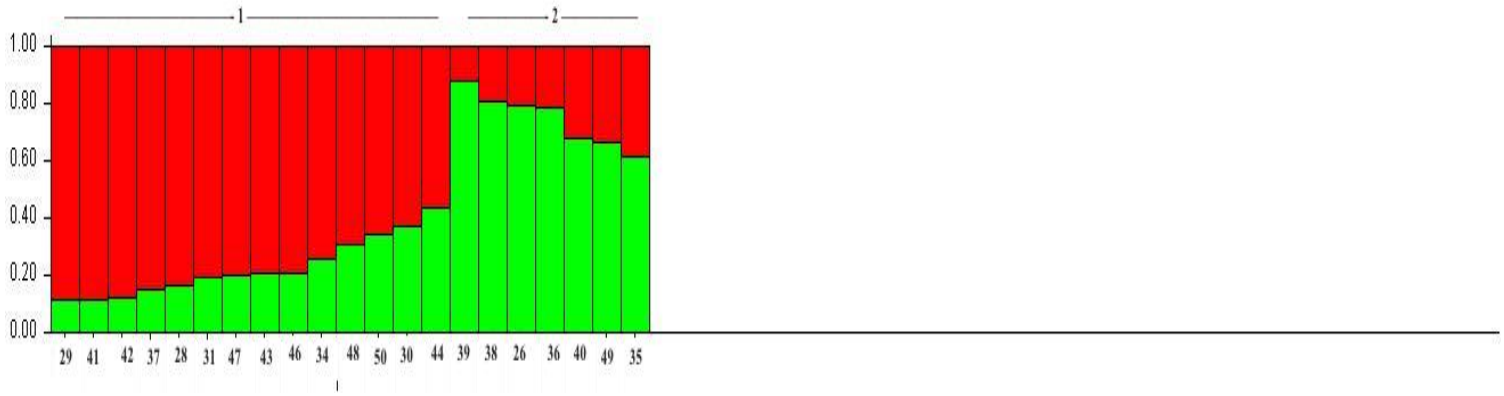

Figure 3.7. Bayesian cluster analysis using *Schizachyrium scoparium* samples from New England. Numbers on labels correspond to sample number on dendrogram.

CHAPTER 4

Attractiveness of Species of *Vitex* (Chastetree) to Native Pollinators[3]

[3] Hawkins, S.M. and C.D. Robacker. Submitted to *HortScience*.

Abstract

Native and non-native bees are important pollinators of both food and ornamental crops. However, bee populations across the world have declined, mainly through loss of habitat. Careful selection of landscape plants in urban areas can help mitigate habitat loss and create new habitat for pollinators. Ten mature genotypes of *Vitex*, comprising *V. agnus-castus* L., *V. negundo* L., and a hybrid between *V. agnus-castus* x *V. rotundifolia* L. f., were evaluated during June and July 2016 to assess their attractiveness to both native and non-native pollinators. Pollinator counts were taken two times daily, at 9:00 a.m. and 11:00 a.m., twice weekly for three weeks. Pollinators were also captured from the *Vitex* plants for identification. The general population of insects in the field was assessed by collecting them in field traps. Insects captured from *Vitex* plants and in field traps were identified to genus and bumblebees [*Bombus* spp. (Latreille, 1802)] were further identified to species. The composition of the mix of pollinators captured in the field traps was different than the mix of pollinators captured on plants. The predominant pollinators captured in field traps were flies (Diptera spp.), ants (*Formicidae* spp.), and sweat bees [*Halictus* spp. (Latreille, 1804) and *Lasioglossum* spp. (Curtis, 1833)] while pollinators captured on the *Vitex* plants were principally bumblebees and honeybees [*Apis mellifera* (Linnaeus, 1758)]. *V. agnus-castus* plants attracted more bumblebees and other native bees than honeybees, while *V. negundo* plants attracted more honeybees than native bees ($p = 0.0008$). *V. negundo* and the *V. agnus-castus* x *V. rotundifolia* hybrid attracted more pollinators over the course of the study than *V. agnus-castus* ($p < 0.0001$). Our study shows that *Vitex* plants can be a good resource to support pollinators in an urban landscape.

Pollinators contribute both economically and ecologically to the regions in which they live. An estimated 87.5% of flowering plants globally, approximately 308,000 species, are pollinated by bees and other animals (Ollerton et al., 2011). Both honeybees (*Apis mellifera*) and wild bees, such as bumblebees (*Bombus sp.*), are important pollinators of food crops. The value of food crop pollination services in the United States was estimated to be approximately 14.6 billion dollars per year in 2009 (Koh et al., 2016). Wild bees were estimated to provide 20% of the food crop pollination services provided (Koh et al., 2016). Pollinators have also been shown to increase the value of ornamental plants such as holly by increasing berry production (Ollerton et al., 2016).

Unfortunately, populations of honeybees and native bees such as bumblebees have declined in recent years. Both native bees and honeybees have been exposed to pesticides such as neonicotinoids, pathogens such as *Nosema* (Nägeli, 1857), and pests such as varroa mites which have all caused population declines (Cameron, 2011; Goulson, 2015). Habitat loss is also considered to be a significant reason for the decline of bee populations (Brown and Paxton, 2009; Goulson, 2015). Conversion of natural habitat to agricultural production and fragmentation of the landscape by cities and suburbs has decimated habitat for nesting and destroyed food sources for bees (Potts et al., 2010). Fragmented habitat can lead to smaller bee populations with decreased genetic diversity and less resistance to pests and pathogens (Cameron, 2011). Although most pollinator studies have been on the major pollinators, such as honeybees and bumblebees, habitat loss, pesticides, pathogens, and pests may also impact lesser studied pollinator species.

The world is becoming increasingly urban. In 2014, 54% of the world's population was estimated to live in urban areas; the figure is estimated to be 66% by 2050 (United Nations, 2014). With increasing development comes a loss in bee species richness (Hernandez et al., 2009). However, incorporation of plants that support pollinators in urban landscapes may help mitigate habitat loss.

Urban areas had a higher number of bee species in studies comparing urban and agricultural areas (Senapathi et al., 2017). Bumblebees in urbanized areas had higher reproductive fitness and colonies contained more stored food than those in agricultural areas in a study comparing cities, villages, and agricultural areas in England (Samuelson et al., 2018). The composition of plant species in urban areas has a large effect on the area's ability to support pollinators. In a study of the richness of pollinator species in New York City, neighborhood areas planted with cultivars of common horticultural species such as *Petunia* Juss. and *Hydrangea* L. were less attractive to pollinators than nearby greenspaces, which had a more varied mix of plant species (Matteson et al., 2013). Clearly, careful consideration must be given to the choice of species and cultivar when planning urban gardens when the goal is to provide habitat for pollinators.

Species of *Vitex* range from small shrubs to large trees and have long been used as ornamental plants in the landscape (Rani and Sharma, 2013). The genus *Vitex* is the largest in the Verbenaceae family (Rani and Sharma, 2013). Species of *Vitex* are distributed in Asia, India, the Mediterranean, Pakistan, Sri Lanka, and Southern Europe (Rani and Sharma, 2013). Both *Vitex agnus-castus* and *V. negundo* have been used as honey plants (Dogan et al., 2011; Harugade et al., 2016). Flowers of *Vitex* species are attractive to a wide range of pollinators, including honeybees, bumblebees, and butterflies (Ashoke and Sudhendu, 2012; Jain, 2013; Murren, 2014; Reddy et al., 1992).

Since *Vitex* is not native to the United States, the question remains as to whether it is a good choice for a landscape plant to support native bees as well as honeybees. Exotic plants have been shown to be detrimental to some native pollinators (Wilde et al., 2015). Species abundance was four times greater on sites planted with native plants than on sites planted with exotic plants in a study

of Lepidopterans in suburban landscapes in Pennsylvania (Burghardt et al., 2009). However, Stout and Morales (2009) concluded that exotic plants could support generalist pollinators in a landscape with few other floral resources. The native status of a plant had no significant effect on attractiveness to pollinators in a study of native and cultivated varieties of flower species in Britain (Garbuzov and Ratnieks, 2014). Modifications of floral morphology through plant breeding, such as double flowers, had more effect on attractiveness to pollinators than native status in studies of garden flowers in Britain (Corbet et al., 2001; Garbuzov and Ratnieks, 2014). Attractiveness of an exotic plant species to pollinators will vary with the species and must be evaluated on a case-by-case basis.

In this study, our primary objective is to evaluate the attractiveness of *Vitex* to pollinators. As part of this objective we sought to answer the question: are native pollinators attracted to *Vitex*?

Materials and Methods

The study was conducted during the months of June and July 2016 in a field plot at the University of Georgia campus in Griffin, GA. Ten mature genotypes of *Vitex* were selected (Table 4.1, Fig. 4.1). Six were genotypes of *V. agnus-castus*, three were cultivars or selections of *V. negundo*, and one was an interspecific hybrid between *V. agnus-castus* and *V. rotundifolia*. All genotypes were in full bloom at the beginning of the study. The composition of plants in and immediately surrounding the field plots was *Abelia spp*. R. Br. (in bloom); other woody ornamental plants (not in bloom); grasses (both cultivated and wild) and weeds; and mixed hardwood forest (behind the field plot). The University of Georgia Research and Education Garden was located northeast of the field plot, across a road and approximately 403-m distant in a straight line, and contained mixed annuals, perennials, and woody ornamentals, some of which were in bloom at the time of the study. Also, located in the Research Garden were several hives of

honeybees. The *Vitex* field plot consisted of 9 rows spaced 4.6-m apart. Each row contained 24 *Vitex* plants spaced 2.4-m apart. *V. agnus-castus* plants used in the study were toward the north end of the plot and *V. negundo* plants toward the south end. The *V. agnus-castus* x *V. rotundifolia* hybrid was approximately in the middle of the plot.

Pollinators were counted twice a day at 9:00 and 11:00 AM. Morning hours were chosen based on prior observation of pollinators in the field and information from prior studies (Jain et al., 2013; Gurel et al., 2008). Counts were repeated twice a week for three weeks. Counts were discontinued when the peak blooming period was substantially over. Pollinators on each plant were counted for a three-minute period on opposite sides of the plant. Following the counts, pollinators were captured by placing a 3.8-L plastic bag over a randomly selected inflorescence containing at least one pollinator, closing the bag with the pollinator inside, and sealing it. Five bags per plant per count period were collected when possible. Captured insects were killed by placing the plastic bags in a freezer at 0°C. Insects were stored in the freezer and later identified to genus with a Wild MPS545 stereomicroscope (Wild Heerbrugg Ltd., Heerbrugg, Switzerland). Identifications were made by the first author with the aid of a reference collection provided by the Department of Entomology at the University of Georgia. Voucher specimens were identified by Conor Fair of the Department of Entomology at the University of Georgia. Voucher specimens were deposited with the Museum of Natural History, University of Georgia, Athens, GA.

To compare the general population of insects in the field to the pollinators captured on the *Vitex* plants, field traps constructed from yellow plastic bowls 15-cm in diameter were filled with a mixture of water and a small amount of dishwashing liquid (Woodcock et al., 2013). The traps were placed at 20-m intervals throughout the field for a 48-h period each week of the study. At the

end of the 48-h period, the liquid in the traps was strained through fiberglass insect screening (Phifer, Inc., Tuscaloosa, AL) and the insects that had been captured in the liquid were transferred from the screening into a plastic bag which was stored in a freezer at 0°C. Captured insects were later identified to genus with a Leica MZ6 stereomicroscope (Leica Microsystems Inc., Buffalo Grove, IL).

Captured insects were identified to five main categories: honeybees, bumblebees, carpenter bees [*Xylocopa* spp. (Latreille, 1802)], sweat bees (*Halictus* spp. and *Lasioglossum* spp.), and other small pollinators. Pollinators in the genus *Bombus* were further identified to species. Captured insects were also classified as belonging to either native or non-native genera. After identification, captured insects were stored in a freezer in the laboratory at 0°C. Data for captured insects were pooled over dates and times.

Flower size was determined for each of the genotypes in the study. Ten flowers from each plant were collected. Flower length was measured from the bottom of the corolla tube to the tip of the corolla. Flower width was measured across the widest part of the corolla. A flower area was calculated by multiplying width times length.

Data were analyzed with SAS 9.3 (SAS Institute, Inc., Cary, NC) using proc glimmix. A log link function was used for count data. Transformed data were backtransformed for presentation. Data were analyzed as a repeated measures experiment and date was treated as a random effect. Means separation was performed using Tukey's HSD ($P < 0.05$) for differences within treatment method and confidence intervals were calculated for means.

Results and Discussion

Of the insects captured in traps in the field, 60% belonged to species that were pollinators or potential pollinators and 40% were non-pollinating insects. The composition of the population of pollinating species captured in the traps was quite different than

that of the population captured on the *Vitex* plants (Table 4.2). Flies comprised 57% of the pollinating species, followed by ants at 15% (Table 4.2). Both genera of sweat bees combined accounted for 12% of the pollinators captured in the field traps (Table 4.2). No honeybees or carpenter bees were captured in the traps (Table 4.2). Bumblebees comprised 0.7% of the total captured in the traps (Table 4.2). In contrast, bumblebees accounted for 52% of the pollinators captured on the *Vitex* plants (Table 4.2). Honeybees comprised 25% of pollinators captured on the plants followed by carpenter bees at 12.5% (Table 4.2). Both genera of sweat bees combined accounted 8% of the population of pollinators captured on the plants (Table 4.2).

Honeybees were considered to be non-native pollinators while all other species identified were native pollinators. Tukey means separation showed that *Vitex* species had different mixes of native and non-native pollinators ($p = 0.0008$) (Table 4.3). *V. agnus-castus* attracted more native pollinators than honeybees, while *V. negundo* attracted more honeybees than native bees. The *V. rotundifolia* x *V. agnus-castus* hybrid attracted similar numbers of native pollinators and honeybees.

Tukey means separation showed that pollinator types captured in the field were different among the *Vitex* species and the hybrid ($p = 0.0008$). However, means separation showed that no interaction existed between pollinator type and the species of *Vitex* upon which it was captured ($p = 0.0541$) (Table 4.4). *Vitex* species and the hybrid all had similar numbers of native pollinators.

Differences in floral morphology may help explain pollinator preferences among species in the same genus. Flowers of *V. negundo* were shorter than those of either *V. agnus-castus* or the *V. agnus-castus* x *V. negundo* hybrid (Table 4.1). A flower with a shorter corolla tube, such as *V. negundo*, might be more attractive to a short-tongued pollinator like the honeybee than a species with a longer corolla tube such as *V. agnus-castus*. Smaller pollinators preferred smaller-flowered species of *Dalechampia* L., while larger

pollinators favored larger-flowered *Dalechampia* (Armbruster and Herzig, 1984). Lavender (*Lavandula* x*intermedia* Emeric x Loisel. 'Grosso') was pollinated far more frequently by bumblebees while honeybees were the common pollinator on borage (*Borago officianalis* L.), which had shallower flowers (Balfour et al., 2013). In the Cambridge University Botanic Garden, UK, honeybees were attracted to shallower flowers than were bumblebees during an evaluation of twenty-four plant species for usefulness to pollinators as nectar sources (Comba et al., 1999).

Attractiveness of *Vitex* plants to pollinators may also be driven by other factors than corolla tube length, such as the scent of the flowers or the amount of nectar available. In a study of honeybees, bumblebees, and carpenter bees on *Agave schottii* Engelm., more honeybees occurred on plants and in sites producing high amounts of nectar, while carpenter bees were present on plants and in sites producing the lowest amount of nectar (Schaffer et al., 1979). Bumblebees occurred on plants and in sites that were intermediate for nectar production (Schaffer et al., 1979).

Attraction of pollinators to a plant may be driven by competition among pollinator species. A follow-up study of pollinators on *Agave schottii* determined that honeybees dominated patches of plants with high nectar productivity, effectively shutting out bumblebees until the nectar supply had been depleted (Schaffer et al., 1983). Honeybees outcompeted native bees of the genus *Andrena* (Fabricius 1775) on apple trees (*Pyrus malus* L.) in an old field in New York state (Ginsberg, 1983). Foraging populations of two bumblebee species increased in number when honeybees were absent during the second year of a pollinator study in mountain meadows of the Rocky Mountains (Pleasants, 1981). Since bumblebees and other native pollinators were present in similar numbers

on each species or hybrid of *Vitex* plants regardless of the number of honeybees present, competition from honeybees did not seem to affect native pollinators in our study.

The number of pollinators we counted during our study varied among species and the hybrid of *Vitex*. Genotypes of *V. agnus-castus* had fewer pollinators than those of *V. negundo* or the *V. agnus-castus* x *V. rotundifolia* hybrid ($p < 0.0001$) (Table 4.5). Differences in attractiveness to pollinators among species and among genotypes within species are not uncommon. Two milkweed species, *Asclepias exaltata* L. and *A. syriaca* L., as well as their hybrid, attracted different numbers and types of pollinators (Stoepler et al., 2012). Attractiveness to pollinators varied among cultivars in a study of a crape myrtle species, *Lagerstroemia indica* L., and *L. indica* x *L. faurei* Koehne hybrids, although all cultivars supported both native and non-native bees (Riddle and Mizell, 2016). Time of day of data collection made no difference in pollinator count ($p = 0.0645$).

Conclusion

Our study shows that *Vitex* can be a good addition to the landscape to help support native pollinators, such as bumblebees. A study of pollinators on both exotic and native species concluded that exotic plant species provided support for native pollinators, especially solitary bees, by providing additional floral resources and extending the time that floral resources are available to bees (Salisbury et al., 2015). When pollination networks in diverse areas of the world were examined, pollinator species richness was greater in locations containing exotic plant species as well as native species (Stouffer et al., 2014). Memmot and Waser (2002) showed that exotic plants could successfully integrate into the native plant-pollinator network, although the effects on native plants might be mixed. The addition of carefully selected non-native plants may enrich floral resources and lengthen bloom time in urban areas. Non-

native plants such as *Vitex* may help mitigate fragmentation of landscape and habitat loss, providing much-needed support to pollinators.

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Table 4.1. Genotypes, parentage, and morphological characteristics of *Vitex* used in the pollinator study.

Genotype	**Species or parentage**	**Flower color**	**Plant height (cm)**	**Plant width (cm)**	**Flower width (mm) (CI)[z]**	**Flower length (mm) (CI)[z]**	**Flower area (mm^2) (CI)[z]**
V. Danica Pink™	*V. agnus-castus*	Pink	250.0	400.0	4.7 [4.0, 5.3]	6.6 [6.1, 7.1]	31.0 [26.6, 35.4]
V. Pink Pinnacle™	*V. agnus-castus*	Pink	353.0	491.5	6.9 [6.2, 7.5]	3.9 [3.4, 4.4]	26.6 [22.2, 31.0]
V. Dale White™	*V. agnus-castus*	White	502.0	458.5	6.7 [6.0, 7.3]	5.9 [5.4, 6.4]	39.1 [34.7, 33.5]
V. Petty Blue™	*V. agnus-castus*	Blue	437.0	484.5	8.0 [7.3, 8.6]	3.0 [2.5, 3.5]	24.0 [19.6, 28.4]
V. 'Salinas Pink'	*V. agnus-castus*	Pink	297.0	450.0	7.5 [6.8, 8.1]	4.4 [3.9, 4.9]	33.3 [28.9, 37.7]
V. 'Silver Spires'	*V. agnus-castus*	White	286.0	393.5	7.7 [7.0, 8.3]	3.6 [3.1, 4.1]	27.4 [23.0, 31.8]

V. negundo heterophylla	*V. negundo*	Blue	313.0	454.5	7.7 [7.0, 8.3]	2.1 [1.6, 2.6]	16.1 [11.7, 20.5]
VHET *negundo*	*V. negundo*	Blue	408.0	487.0	8.0 [7.3, 8.6]	2.0 [1.5, 2.5]	16.0 [11.6, 20.4]
Little Madame	*V. negundo*	Blue	261.0	411.0	7.3 [6.6, 7.9]	2.0 [1.5, 2.5]	14.6 [10.2, 19.0]
V0502-7	*V. agnus-castus* x *V. rotundifolia*	Blue	265.0	364.5	13.3 [12.6, 13.9]	4.0 [3.5, 4.5]	53.3 [48.9, 57.7]

[z]Ten flowers of each genotype were measured for width, length, and area. Confidence limits at alpha = 0.05 are presented in brackets next to flower measurements.

Table 4.2. Composition of the population of pollinators and potential pollinators captured on *Vitex* plants compared to the population captured in traps in the field.

Scientific name	**Common name**	**Count of insects captured on plants**	**Count of insects captured in field traps**	**% of insects captured on plants**	**% of insects captured in field traps**
Apis mellifera	Honeybee	139	0	24.9	0.0
Bombus bimaculatus	Two-spotted bumblebee	3	1	0.5	0.2
Bombus impatiens	Common Eastern bumblebee	279	3	50.0	0.5
Bonbus griseocollis	Brown-belted bumblebee	7	0	1.3	0.0
Coleoptera sp.	Beetles	0	34	0.0	5.6
Diptera sp.	Flies	0	344	0.0	56.9
Formicidae sp.	Ants	2	90	0.4	14.9
Halictus sp.	Sweatbee	9	17	1.6	2.8
Hymenoptera sp	Other small bee	2	8	0.4	1.3
Laphria sp.	Robber flies	1	0	0.2	0.0
Lasioglossum sp.	Sweat bees	37	54	6.6	8.9
Lepidoptera sp.	Moths	0	9	0.0	1.5
Leucospis sp.	Leucospid wasp	9	1	1.6	0.2

Vespidae sp.	Wasps, other	0	44	0.0	7.3
Xylocopa sp.	Carpenter bee	70	0	12.5	0.0
Total		**558**	**605**	**100.0**	**100.0**

Table 4.3. Mean number of native and non-native pollinators captured per plant and sampling period for all genotypes of *V. agnus-castus* and *V. negundo* and for the *V. agnus-castus* x *V. rotundifolia* hybrid with standard error and confidence intervals at alpha = 0.05.

	Native				**Non-Native**			
			95% Cis				**95% CIs**	
Species/Hybrid	**Mean no. of pollinators[z]**	**Std err**	**Lower bound**	**Upper bound**	**Mean no. of pollinators[z]**	**Std err**	**Lower bound**	**Upper bound**
V. agnus-castus	2.3	0.14	2.0	2.6	1.4	0.29	0.9	2.1
V. negundo	1.8	0.19	1.5	2.2	2.8	0.29	2.3	3.4
V. agnus-castus x *V. rotundifolia*	2.5	0.34	1.9	3.2	1.9	0.45	1.2	3.0

[z]N = 9 for *V. agnus-castus*, n=3 for *V. negundo*, and n = 1 for *V. agnus-castus* x *V. rotundifolia* with six sampling dates and two times per sampling date each.

Table 4.4. Mean number of pollinators captured per plant and sampling period for all genotypes of *V. agnus-castus* and *V. negundo* and for the *V. agnus-castus* x *V. rotundifolia* hybrid by type of pollinator with standard error and confidence intervals at alpha = 0.05.

Pollinator			Species/Hybrid of *Vitex*		
			V. agnus-castus	***V. negundo***	***V. agnus-castus* x *V. rotundifolia***
Honeybees		**Pollinators[Z]**	1.4	2.8	1.9
		Std err	0.29	0.29	0.45
	95% Cis	**Lower bound**	0.9	2.3	1.2
		Upper bound	2.1	3.4	3.0
Bumblebees		**Pollinators[Z]**	2.9	2.4	2.8
		Std err	0.22	0.29	0.45
	95% Cis	**Lower bound**	2.1	3.4	3.0
		Upper bound	3.4	3.0	3.9
Carpenter Bees		**Pollinators[Z]**	1.7	1.0	1.8
		Std err	0.22	0.55	0.83
	95% Cis	**Lower bound**	1.3	0.3	0.8

		Upper bound	2.2	3.0	4.5
Sweat Bees		**Pollinators[Z]**	1.4	1.1	1.9
		Std err	0.29	0.30	0.67
	95% Cis	**Lower bound**	1.0	0.6	0.9
		Upper bound	2.1	1.8	3.8
Other small pollinators		**Pollinators[Z]**	1.6	1.3	2.0
		Std err	0.96	0.45	1.40
	95% Cis	**Lower bound**	0.5	0.7	0.5
		Upper bound	5.2	2.6	8.0

[Z]N = 9 for *V. agnus-castus*, n=3 for *V. negundo*, and n = 1 for *V. agnus-castus* x *V. rotundifolia* with six sampling dates and two times per sampling date each.

Table 4.5. Mean number of pollinators per plant for all genotypes of *V. agnus-castus* and *V. negundo* and for the *V. agnus-castus* x *V. rotundifolia* hybrid with standard error and confidence intervals at alpha = 0.05.

			95% Cis	
Species or Hybrid	**Mean no. of pollinators[Z]**	**Std err**	**Lower bound**	**Upper bound**
V. agnus-castus	29.0	1.85	25.5	32.9
V. negundo	58.7	5.11	49.4	69.8
V. rotundifolia x *V. agnus-castus*	53.7	8.13	39.8	72.5

[Z]N = 9 for *V. agnus-castus*, n=3 for *V. negundo*, and n = 1 for *V. agnus-castus* x *V. rotundifolia* with six sampling dates and two times per sampling date each.

Figure 4.1a. Flowers of *V. agnus-castus*

Figure 4.1b. Flowers of *V. negundo*.

Figure 4.1c. Flowers of hybrid of *V. agnus-castus* x *V. rotundifolia*.

Figure 4.1. Flowers of the two species and one hybrid of *Vitex* used in the study.

CHAPTER 5

Conclusions and Next Steps

Little bluestem, with its high genetic and phenotypic variability, is a prime candidate for the continued production of novel ornamental cultivars. Although the majority of genetic variation in little bluestem is within populations, variation was also found among regions in our study. Regional genetic variation should continue to be explored to facilitate the breeding of regionally-adapted cultivars. With the breeding of new cultivars comes the need for a rapid propagation method to clonally produce plants. The micropropagation protocol developed in our study should be useful for growers, as well as researchers.

Vitex is a beautiful, low-maintenance, and tough landscape plant. *Vitex*, although a non-native, proved attractive to native bees in our study. As such, *Vitex* should be a useful plant to support pollinators, especially in urban environments where few floral resources may exist.

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