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Buffalograss Diversity Assessment

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BUFFALOGRASS DIVERSITY ASSESSMENT

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Buffalograss [*Buchloë dactylodes* (Nutt.) Engelm. syn. *Bouteloua dactyloides* (Nutt.) Columbus] is a low input alternative turfgrass for the Great Plains region. Buffalograss is a dioecious stoloniferous warm season grass with wide variation of valuable traits and is well adapted for use in minimal maintenance landscapes. The obligate outcrossing nature of the species and wide variation of potentially valuable traits make buffalograss a prime candidate for cultivar improvement and genetic study. Limited information is available regarding buffalograss genetics. In effort to expand genetic resources around buffalograss, we developed buffalograss derived simple sequence repeat markers from previously available transcriptomes of buffalograss cultivars '378' and 'Prestige'. Simple sequence repeat markers were developed using the microsatellite identification tool (MiSa). We developed 139 simple sequence repeat markers. A panel of 24 of the markers were confirmed for amplification and validated on a set of seven buffalograss cultivars. A collection of 96 new buffalograss genotypes were collected from throughout the Great Plains region and genetically characterized using the panel of SSR markers. The collection was clustered by similarity using the unweighted pair-group arithmetic means (UGMA) produced by the sequential, agglomerative, hierarchical, and nested clustering methods (SAHN) program in the Numerical Taxonomy (NTSYS) system of software, genetic similarity coefficients ranged from 0.46 to 1.00. The collection was also observed for visual quality, establishment, stolon count, stolon width,

genetic color, fall color, and spring green up. Phenotypic differences were observed between genotypes and by geographic location that the genotype was collected.

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LITERATURE REVIEW

Little is known about genetic diversity among and between buffalograss [*Buchloë dactylodes* (Nutt.) Engelm. syn. *Bouteloua dactyloides* (Nutt.) Columbus] accessions. Without fully understanding genetic diversity, it is difficult to maintain unique genetics or limit genetic redundancy in germplasm collections (Cornuet and Luikart, 1996). Currently, the turfgrass breeding program at the University of Nebraska-Lincoln maintains 2986 buffalograss accessions. Many of these lines have been evaluated for quality, color, canopy density, establishment rate, seed yield, uniformity, low mowing tolerance, stolon length, chinch bug resistance, and mealy bug resistance. Many of these accessions were derived from bi-parental crosses of parents already in the collection. There is a risk of wasting resources by maintaining redundant lines or forming genetic bottleneck in a collection if repetitive breeding practices are routinely used. Two approaches to introduce new genetics and genetically characterize the collection are 1) collect and introduce wild, unimproved buffalograss genotypes into a breeding program, and 2) use molecular markers on newly collected and elite buffalograss genotypes to assess genetic diversity. A wide range of genetic diversity allows for focus in improved buffalograss cultivar breeding.

Buffalograss has potential to be used as a native alternative to reduce turf management costs, because of its low water and fertility use, lateral spread, and pest tolerance. Within the Great Plains region, buffalograss has been used as a forage for

wildlife and livestock, home lawns, sports fields, and roadside erosion control. Buffalograss performance in a low input environment, make it a viable turf option for homeowners and turf managers in arid or semi-arid regions (Wenger, 1943). Buffalograss is a valuable turfgrass species, because it is stoloniferous and forms a dense sod; has low fertility requirements; tolerates low mowing; is cold, heat, and drought tolerant; has relatively few pests compared to other species; has acceptable visual quality; and it is a well performing turf (Frank et al., 2002; Goss, 2017; Heng-Moss et al., 2002; Johnson et al., 2001; Johnson-Cicalese et al., 2011; Karcher et al., 2008; Morris, 2003; Qian et al., 2000; Ruemmele et al., 1993; Shearman et al., 2004; Steinke et al., 2011). Considering all of these traits, buffalograss is a valuable turfgrass species in the Midwest and semi-arid regions of North America.

The stoloniferous nature of buffalograss contributes to the formation of a dense sod and quick establishment of buffalograss stands (Giese et al., 1997). Ruemmele et al. (1993) evaluated buffalograss establishment by plugging and sprigging methods to establish buffalograss. Both vegetative establishment methods were evaluated at 1:60 and 1:30 ratios of vegetative sod plugs or washed sod sprigs to bare soil planting area. Neither ratio or method were different from another for establishment and each buffalograss stand reached at least 97% coverage by the end of one year, resulting in either method of buffalograss establishment to be sufficient . When ‘Texoka’ buffalograss plugs were exposed to pre-rooting treatments, Johnson et al. (1997) observed superior coverage by buffalograss stands treated with eight-week pre-rooting treatments at the end of one

establishment year. Two and four-week pre-rooting treatments resulted in faster establishment then untreated plugs. Higher rates of establishment were observed when buffalograss plots were applied with supplemental fertility (Frank et al., 2002).

Frank et al. (2000a, 2000b) found that established buffalograss achieved a high visual turf quality with optimal applications of ammonium nitrate up to 97kg·ha^{-1} to 147kg·ha^{-1}, and a one-time application of 49kg·ha^{-1} phosphorous depending on location (Frank et al.,2000a; Frank et al., 2000b; Frank et al., 2002). Increased fertility was suggested to be detrimental to buffalograss establishment in favor of weed competition (Frank et al., 2002). Established buffalograss requires less supplemental fertility than other common turf species. Buffalograss irrigated at 60% evapotranspiration (ET) replacement, on average recovered 51% of the 49kg·ha^{-1}nitrogen applied from total buffalograss tissues. When 98kg·ha^{-1} nitrogen was applied only 31% of the applied nitrogen was recovered. These results suggest that higher application rates of nitrogen are unnecessary (Frank et al, 2000a; Frank et al., 2000b). Comparatively, when Kentucky bluegrass [*Poa pratensis* (L.)] and tall fescue [*Lolium arundinaceum* (Schreb.)] were irrigated at 80% ET replacement, a range of 63.3% to 89.8% of 49kg·ha^{-1} nitrogen applied was recovered from total Kentucky bluegrass tissues and a range of 52.9% to 81.8% of 49kg·ha^{-1} nitrogen applied was recovered from total tall fescue tissues (Frank, 2000). Prior to nine weeks after seeding, untreated buffalograss plots and those treated with 49kg·ha^{-1} phosphorous showed no differences in percent coverage, but there were differences by 9, 10, and 11 weeks after seeding. Buffalograss stands showed no response to the application of additional potassium, attributed to adequate pre-planting levels

(Frank et al., 2002). These data support that buffalograss has lower fertility requirements than tall fescue and Kentucky bluegrass.

Mowing height and frequency are important to the intrinsic value of a turfgrass. Homeowners and vegetation maintenance contractors can mow buffalograss once per year at typical turf height (1in to 3in) to maintain a "natural" looking buffalograss stand (Riordan et al., 1993). When maintained at reduced mowing heights, buffalograss stands are reported to have decreased weed competition (Riordan et al., 1993). Johnson et al. (2000) found that buffalograss accessions '86-61', '86-120', and ' 315' show vigorous and competitive growth with high quality when mown at 15mm. Plots were established at 7.6cm tall and the mowing height was gradually lowered to 1.6cm over two months and assessed for quality. Once reaching 1.6cm, plots were mowed once per week during the spring and end of the summer of 1995, and twice per week during peak growing activity. When mowed at 1.6cm there was a negative relationship between summer quality and fall color (Johnson et al., 2000). Johnson explains that when mown at a low height, buffalograss must be well adapted to the area it is being grown, or it will be subject to winterkill. In the third part of this study, female lines had a higher visual quality than male and monecious lines. A wide ranges of mowing height tolerance has been reported in buffalograss ranging from 15mm to 100mm depending on the genotype. Low mowing tolerance may be maternally inherited. At low mowing height, progeny of female buffalograss '86-61', the best low mowing tolerance line, exhibited the highest average visual quality (Johnson et al., 2000). Buffalograss tolerance to low mowing heights make

it a viable option to be used as golf course fairways (Johnson et al., 2000; Riordan et al., 1993).

Buffalograss is winter hardy and can survive prolonged exposure to sub-zero temperatures by going dormant (Wegner, 1943). Once temperatures begin to decrease from fall to winter, buffalograss loses its color and begins to go dormant. Qian et al. (2001) found that 'UCR', '91-118', 'Stampede', and '609' buffalograss were tolerant of freezing temperatures down to -12°C and northerly adapted cultivars 'Tatanka' and 'Texoka' were tolerant of temperatures down to -20° . Low-temperature tolerance was defined as 50% or more survivability at the time of evaluation. Data were collected monthly from September 1998 to April 1991, and October 1999 to May 2000 (Qian et al., 2001). Differing cold tolerance levels are attributed to the differing abilities to acclimate to the cold. Initiation of acclimation occurs by changes in day length and temperature. There were no differences in breaking winter dormancy and spring green-up among the cultivars tested. (Qian et al., 2001).

Buffalograss is a drought tolerant turfgrass species. Buffalograss has comparable evapotranspiration rates to bermudagrass [*Cynodon dactylon* (L.) Pers.], seashore paspalm (*Paspalum vaginatum* Swartz.), zoysiagrass (*Zoysia japonica* Steud), centipedegrass [*Eremochloa ophiuroides* (Munro) Hack], and St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] (Huang, 2008). The ET rate for buffalograss over a two-year period was 4.76mm d^{-1} and is less than the pooled average ET rate of

5.37mm d^{-1} for the species above and performed in the lowest ET rate grouping (Kim and Beard, 1988). In Las Cruces New Mexico, Goss et al. (2017) compared the water use of buffalograss to other species. Buffalograss required 64% less irrigation than bermudagrass, 79% less irrigation than tall fescue [*Lolium arundinaceum* (Schreb.)], and 83% less irrigation than Kentucky bluegrass [*Poa pratensis* (L.)] to maintain 100% turf cover. In order to maintain 50% cover, buffalograss required 73%, 85%, and 88% less irrigation than bermudagrass, tall fescue, and Kentucky bluegrass, respectively (Goss et al., 2017). Buffalograss low consumptive water use is attributed to a deep root system and large root to shoot ratio (Qian et al., 1997).

In San Antonio, TX, buffalograss and bermudagrass were tested for drought tolerance in an unrestricted planting depth, buffalograss '609' reached 50% green cover in 2006 after 35 days without water, and 60 days to reach 50% green cover in 2007. Bermudagrass required a range from 20.3 to 50 days to reach 50% green cover in 2006, and 33 to > 60 days to reach 50% green cover in 2007 (Steinke et al., 2011). However, when the roots of the plant were restricted to a 10cm depth, no plants survived 60 days without supplemental water. When subjected to drought both buffalograss and bermudagrass began to lose green color by day 15 (Steinke et al., 2011). After 60 days of drought, drought recovery was then tested. The site was prepared by first removing the brown turf canopy by mowing and then irrigating the site at 1.3cm of water every three days. In 2006 and 2007, buffalograss recovered to 50% green cover in 8.1 days and 12.5 days respectively. In the same study, bermudagrass required a range of 14.6 and 14.9

days with water to reach 50% green cover in 2006 and 2007 respectively (Steinke et al., 2011). Low ET rates of buffalograss had a positive relationship with leaf area and a negative relationship with trichome number (Kim and Beard, 1988). High trichome count is related to higher rates of susceptibility to arthropod pests (Johnson-Cicalese et al., 2011).

Buffalograss is host to a relatively low number of arthropod pests but this list includes mound building prairie ants [*Pogonomyrmex occidentalis* (Cresson)], buffalograss webworm [*Surattha identella* (Kearfott)], eriophyid mite [*Eriophyes slykhuisi* (Hall)], fall armyworm [*Spodoptera frugiperda* (J.E. Smith)], white grubs [*Phyllophaga crinite* (Burmesiter)], mealybugs [*Antionia gaminis* (Maskell) and *Tidiscus sporoboli* (Cockrell)], and chinch bugs [*Blissus occiduus* (Barber)] (Heng-moss et al., 2002; Wenger, 1943). Mealybugs and chinch bugs have been the focus of numerous studies and have been identified as important pests causing significant damage to buffalograss (Heng-Moss et al., 2002). Insecticides are one method to control these pests; however, buffalograss' high threshold of feeding tolerance offers another control option. Heng-Moss et al. (2002) reports variation in the level of chinch bug damage in different buffalograss cultivars. Cultivars most susceptible to chinch bug feeding damage were '315' and '378', and 'Tatanka'; 'Bonnie Brae', 'Prestige, and 'Cody' were highly or moderately tolerant to feeding damage after being heavily infested with chinch bugs. Heng-Moss et al. (2002) also reports that seeded varieties such as 'Cody' and 'Tatanka' showed variation in chinch bug feeding damage, likely due to the higher amount of

genetic diversity in seeded varieties compared to vegetative varieties. Johnson-Cicalese et al. (2011) reported that ploidy level, trichome number, and resistance to mealybugs may be related. Buffalograsses with a greater trichome concentration such as ’85-97’ and ‘85-217’ show high susceptibility to mealybug infestation, whereas tetraploid cultivars ‘609’ and ‘84-412’ with a lower trichome density were highly resistant (Johnson-Cicalese et al., 2011).

Buffalograss is theorized to have originated in the warm region of central Mexico and southern Texas (Quinn, 1991). Northward migration of the species from Central Mexico to Canada falls within the same habitat as large migratory herbivores (Quinn et al. 1994). Buffalograss possesses a strong winter dormancy response and is found naturally occurring in the regions of Wyoming, Montana, and Canada. Buffalograss is a sub-dioecious, stoloniferous, perennial grass species suitable for use as a turfgrass. Buffalograss is most suited to areas within the Great Plains from Illinois west to Wyoming, because of its low water and fertility requirements. Buffalograss has a pointed leaf shape, blueish green hue, and pubescent ligule and leaf. Staminate flowers are spike shaped and extend above the canopy on a culm ranging from ten to thirty centimeters, facilitating wind pollination (Huff and Wu, 1992; Quinn, 1991). Pistilate flowers are arranged in a panicle and are found within the canopy on culms three to seven centimeters long. Buffalograss florets are often described as "burr-like’ and develop into a burr containing 3-5 caryopses (Huff and Wu, 1992). Quin et al. (1994) states that the burrs are viable after passage through cattle, facilitating migration of the species with the

animal. Because of its dioecious nature, buffalograss is highly heterogeneous complicating plant breeding efforts to fix desirable traits. The dioecious nature of buffalograss is attributed to convergent evolution where it is believed to have evolved from a monecious state, facilitating an outcrossing advantage (Conner, 1981; Quinn, 1991). Buffalograss uses the C4 photosynthesis pathway, allowing the plant to be more water efficient than C3 photosynthetic plants and makes buffalograss better adapted to warm dry regions. Buffalograss represents a ploidy series and diploids, tetraploids, pentaploids, and hexaploids have been reported (Budak et al., 2004b). Diploids and tetraploids are more southerly distributed whereas hexaploids and pentaploids are distributed, and better adapted, to cooler regions of the northern Great Plains (Johnson et al. 1998, Johnson et al., 2001).

Organelle gene sequences and nuclear molecular marker technologies have been used to assess buffalograss ploidy, geographical distribution, taxonomic placement, and genetic architecture (Budak et al., 2004a; Budak et al., 2005b; Columbus et al., 2000; Gulsen et al., 2005a). Buffalograss male and female plants were initially identified as two separate species, *Sesleria dactyloides* (Nutt.) and *Athenphora axilliflora (*Steud), but the two species were reclassified to one species when a sample containing both male and female flowers was discovered by Engelmann in 1859 (Huff et al., 1992). Currently, there are two botanical names for the species, *Buchloë dactyloides* (Nutt.) Engelm. and *Bouteloua dactyloides* (Nutt.) Columbus. In 1999, the monophyletic satellite clade of *Buchloë* was suggested to be a satellite genus of the larger clade of *Bouteloua*

(Columbus, 1999), due to the species dimorphic spikelets, and molecular analysis based on DNA sequence of the internal transcribed spacer (ITS) and *trn*L-F intergenic spacer regions (Columbus et al., 2000). ITS analysis revealed that *Buchloë* is closely related to the species *Bouteloua diversispicula* Columbus *(Cathestecum brevifolium* Swallen*)*, *Bouteloua multifida* (Griffiths) Columbus [*Griffithoschola multifida* (Griffiths) Pierce], *Bouteloua scabra* (Knuth) Columbus [Pentarrhaphis *scabra* (Knuth)], and *Bouteloua Mexicana* (Scribn.) Columbus [*Soderstromia Mexicana* (Scribn.) Morton]. DNA sequence similarity of the *trn*L-F phylogeny and ITS analysis report a similar evolutionary history. Although both botanical names are accepted in the literature the reclassification of *Buchloë dactylodes* (*Nutt.*) Engelm. to *Bouealoua dactyloides* (*Nutt.*) Columbus does generate confusion.

Other molecular markers have been used to assess buffalograss genetics. Restriction fragment length polymorphism (RFLP) technology was used on chloroplast and mitochondrial DNA to elucidate an evolutionary history based on low numbers of mutations in these plastid genomes (Gulsen et al., 2005a). RFLP was the first developed sequence profiling technique and works by using restriction enzymes to digest amplified products, using a known sequence as a hybridization probe, and comparing differences in sequence length on an agarose gel (Powell et. al 1996). RFLPs have been used to study chloroplast and mitochondrial diversity in buffalograss, finding that chloroplast DNA is maternally inherited and similar to blue grama (Gulsen et al., 2005a). Gulsen et al. (2005a) also reports that genetic diversity and ploidy level have no relationship, and that low-level polymorphism rates may be caused by few female origins. When using nine

RFLPs on buffalograss, blue grama [*Bouteloua gracilis* (H.B.K.) Lag. Ex Steud], and perennial ryegrass [*Lolium perenne* L.], a genetic similarity (GS) of 0.64 was found between buffalograss and blue grama. This result is consistent with the placement of buffalograss within *Bouteloua* found by Columbus (1999). Budak et al. (2005) sequenced the chloroplast genes *matK*, *rbcL*, and the mitochondrial gene cytochrome b (*cob*) of buffalograss, blue grama, zoysiagrass, and bermudagrass. Most plastid PCR primers did not amplify in the outgroup species, suggesting DNA sequence diversity between these plastid genomes. Buffalograss and blue grama shared the most amplified markers, furthering the evidence for *Buchloë* being a monophyletic clade in *Bouteloua*. Sequence analysis of *cob* also showed that separate and genetically diverse lines were not necessarily geographically distant in origin, and differences in genotype may be due to selection during breeding.

Random amplified polymorphism DNA (RAPD) technology uses polymerase chain reaction (PCR) to amplify DNA at a single locus ranging from five to one-hundred base pair sequences generally located at the end of a chromosome and have been used in buffalograss phylogenetics (Budak et al., 2004a; Wu and Lin, 1994). Budak et al. (2004a) produced 210 RAPD markers and 79% were found to be polymorphic in buffalograss and each polymorphic band was shared between at least two genotypes. With a coefficient of variation (CV) threshold held at 9.84% for RAPD markers, unweighted pair-group arithmetic mean cluster analysis UPGMA clustered the tested 15 buffalograss genotypes

from this study into five clusters. Comparing ISSSR, SSR, SRAP, and RAPD marker technologies tested in the Budak et al. (2004a) study, RAPD technology had the least distinguishing power, but could identify extremes between seeded and vegetative genotypes.

Inter-simple sequence repeat (ISSR) markers target repeated nucleotide sequence motifs by amplifying the sequence between two repetitive sequences. ISSR technology has been used for genome mapping, diversity assessment, and genetic fingerprinting (Godwin et al., 1997). Other species sequence data can be used to develop ISSR markers, thus no previous sequence data is required to perform ISSR analysis. Budak et al. (2004a) used 30 ISSR markers developed from maize, sorghum, pearl millet and sugarcane to study the genetic relationships between 15 buffalograsses. A panel of 251 ISSR markers were tested and 81% were found to be polymorphic. ISSR analysis clustered buffalograss genotypes into seeded and vegetative biotypes and estimated a cophenetic correlation of r= 0.90 when clustering of the accessions (CV = 10.87%). The average genetic similarity found with ISSR technology was 51%. Clustering of these genotypes show that similarity of genotype was not dictated by geography (Budak et al., 2004a).

Exploiting repetitive genetic sequences, simple sequence repeat (SSR) technology offers a simple process using forward and reverse primers flanking a target DNA sequence of 2-20 motifs ranging one to six base pairs longs. SSRs are codominant, polymorphic, and distributed across the plants' genome, making it a viable option for

genetic assessment, taxonomic studies, and line purity maintenance (Powell et al., 1996; Warnke et al., 2017). SSRs can be detected using agarose gel electrophoresis or newly developed high throughput screening methods (Warnke et al., 2017). SSR markers are transferable from related plant species, and can be developed from transcriptomic RNA or DNA sequence data. The SSR primers must be very specific in nucleotide make up and melt-temperature, and chromosomal location so that they do not mis-amplify and do not create a hairpin or other secondary structures (Budak et al., 2004a; Li and Quiros, 2001). Eighty-six percent of the SSR markers that were reported in the Budak et al., 2004 study derived from maize (*Zea mays* L.), sorghum [*Sorghum bicolor* (L.)], pearl millet (*Pennisetum americanum* L.), and sugarcane (*Saccharum officinarum* L.) were polymorphic in buffalograss. Of the 180 SSRs derived from maize 63% amplified, 79% of the 180 sorghum markers amplified, 82% of the 66 pearl millet markers amplified, and 77% of the 26 sugarcane markers amplified. Buffalograss derived SSRs have been developed from buffalograss sequence data and accurately assess ploidy in buffalograss but have yet to be used to assess genetic diversity (Hadle et al., 2016; Hadle et al., 2019). SSR and ISSR studies agree that there is greater genetic variation within seeded buffalograss genotypes than vegetative genotypes, which is consistent with expectations based on the outcrossing nature of buffalograss.

Sequence related amplified polymorphism (SRAP) technology has been used for molecular characterization in buffalograss and has a high correlation with morphological trait variation (Zhou et al., 2011). SRAP markers are another PCR based marker system

that utilize 17-18bp GC-rich forward and AT-rich reverse primers pairs to gain information of open reading frame sequences (Robarts and Wolfe, 2014). Budak et al., (2004) tested 34 primer combinations on seven genotypes of buffalograss, and on average five alleles were found per primer pair but ranged from one to nine alleles. Polymorphisms were found in 95% of the 263 amplified SRAP markers. Only SRAP technology had the power to uniquely identify genotypes from the same geographic origins when testing 150 clonal propagates from 15 genotypes (Budak et al., 2004b). When testing 288 primer combinations, 207 combinations resulted in 2690 amplified fragments including a female specific segment in buffalograss (Zhou et al. 2011).

In a combined analysis, ISSR, SSR, RAPD, and SRAP markers clustered buffalograss genotypes into four groups by an Unweighted Pair Group Method with Arithmetic Mean with a cophenetic correlation of r=0.94 and a mean similarity of 0.59. This analysis found ‘Cody’ and ‘Density’ to have the greatest genetic distance and placed these cultivars as extremes between seeded and vegetative genotypes. There was a linear response between allele copy number and sample ploidy. Comparatively, RAPD markers did not show variation in allele copy number with change in ploidy level (Budak et al., 2005b). Quickly and efficiently grading buffalograss markers remains a challenge surrounding buffalograss genetic research.

High throughput RNA and DNA sequencing studies have been done in buffalograss to study gender expression, and chinch bug and leaf spot resistance.

However, these high throughput methods have primarily been used to identify changes in gene expression associated with the trait being studied (Amaradasa and Amundsen, 2016; Wachholtz et al., 2013). After RNA sequencing, Amaradasa and Amundsen (2016) found that between two cultivars of differing degrees of leaf spot resistance 461 transcripts were differentially expressed. Rt-PCR primers were produced from the resulting transcript data and are a resource for identification of pathogen resistant buffalograsses. Wachholtz et al. (2013) sequenced the transcriptomes of buffalograss cultivars 'Prestige' and '378' cultivars, resulting in 121,288 expressed transcripts and 325 differentially expressed transcripts between the cultivars. Both studies used a de novo transcriptome assembly approach to create representative transcriptomes that are now available on NCBI, greatly increasing previously available genetic resources. Sequence data from these high throughput sequencing studies can be data mined and used to develop buffalograss-specific molecular markers and assess buffalograss genetic diversity.

The amount of genetic diversity in the University of Nebraska-Lincoln turfgrass breeding program's buffalograss collection has not been recently characterized. Continual breeding for desirable turf traits may have reduced genetic diversity in the collection. Introduction of wild material from the primary growing region would assure diversity of the collection and potentially introduce novel traits. The collection also serves as a panel representing the amount of genetic diversity among wild buffalograss which can be compared to the accessions maintained by the breeding program. The goal of this research is to increase, test, and assess the amount of genetic and phenotypic diversity in

wild and elite buffalograss genotypes within the buffalograss breeding program at the University of Nebraska. Achievement of this goal will expand the available genetic resources surrounding buffalograss genetics and breeding, as well as increase underrepresented buffalograss genotypes within the collection. To support this goal we plan to develop, test, and validate buffalograss derived simple sequence repeat markers using previously available transcriptome data, high-throughput screening methods and collect buffalograss genotypes. The primary objectives of these studies are to; 1) develop an in-silico method of identifying buffalograss derived simple sequence repeat markers generated from previous genetic sequence data, creating a quick and efficient marker development process; 2) simple sequence repeat markers will be tested and validated using high resolution melt analysis, reducing time and labor costs for genetic assessment; 3) collect plants from underrepresented wild buffalograss populations to introduce new genetics into the University of Nebraska Lincoln's buffalograss breeding program; 4) assess buffalograss genetic diversity among and within wild collections, elite breeding lines, and named cultivars, in effort to inform future buffalograss cultivar development and line purity maintenance; and 5) perform a phenotypic evaluation of wild buffalograss genotypes and named cultivars, measuring lateral spread, stolon count, stolon width, internode length, visual quality, genetic color, fall color, and spring green up. These phenotypic traits may be valuable to buffalograss breeders in the development of future buffalograss cultivars.

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CHAPTER ONE

SIMPLE SEQUENCE REPEAT MARKER DEVELOPMENT AND SCREENING USING TWO BUFFALOGRASS TRANSCRIPTOMES

Abstract

Buffalograss is a stoloniferous low input turfgrass species native to the central Great Plains of the United states. Multiple molecular markers techniques have been used to compare relationships among unimproved and elite buffalograss [*Buchloë dactyloides* (Nutt.) Engelm.] genotypes, but most rely on random amplification, are transferred from other species, or are developed from limited DNA sequence data. The objectives of this study were to develop buffalograss derived simple sequence repeat markers (SSRs) from buffalograss sequence data using an in-silico bioinformatic pipeline and apply high resolution melt (HRM) analysis to screen the SSRs on buffalograss cultivars. Transcriptomes from buffalograss cultivars '378' and 'Prestige' were mined for SSRs using MIcroSAtellite identification tool (MiSa). SSRs were compared between the two transcriptomes to identify 259 conserved polymorphic SSRs. HRM was then performed using a subset 96 SSR primers pairs on a panel of seven buffalograss cultivars to test for amplification of the SSR markers. The 22 SSRs screened by HRM had allele frequencies ranging from 0.29 to 1.0. These SSR markers and HRM screening method facilitated quick screening and were able to distinguish each of the buffalograss genotypes tested. These buffalograss-specific SSR markers will be useful to study buffalograss genetic diversity, for line purity maintenance, and for marker assisted selection.

Introduction

Buffalograss [*Buchloë dactyloides* (Nutt.) Engelm.] syn. [*Bouteloua dactyloides* (Nutt.) Columbus] (Columbus, 1999) is a stoloniferous, warm-season grass, native to the Great Plains of North America (Shearman et al., 2004). Buffalograss requires minimal supplemental irrigation and fertility. This species is also tolerant of many turf pests, hot and cold temperatures, infrequent mowing, and a relatively low mowing height. (Frank et al., 2002; Goss, 2017; Heng-Moss et al., 2002; Johnson et al., 2000; Johnson-Cicalese et al., 2011; Qian et al., 2001; Ruemmele et al., 1993; Steinke et al., 2011). A priority of turf-type buffalograss breeding programs is to capitalize on its innate performance in low input environments while improving its visual and functional quality.

Quality traits can be improved through plant breeding and the plant breeding process can be accelerated by using molecular markers to select for early targetable traits (Jones et al., 2001). Inter-simple sequence repeats (ISSR), random amplified polymorphic DNA (RAPD), Random Fragment Length Polymorphism (RFLP), sequence related amplified polymorphism (SRAP), and simple sequence repeat (SSR) molecular markers have been previously used in buffalograss for genetic fingerprinting, cultivar maintenance, and phylogenetic analyses (Budak et al., 2004b; Gulsen et al., 2005; Wu and Lin, 1994; Zhou et al., 2011). Buffalograss ISSRs were able to distinguish most of the eight seeded varieties but were unable to distinguish two of the seven vegetative accessions of close geographical origin (Budak et al. 2004a). Gulsen et al. (2005) reported RFLP markers that indicate a single maternal buffalograss origin. Jones et al.

(1997) used dominant RAPD markers in buffalograss for genetic characterization with high reproducibility. SRAP markers could identify female genotypes in buffalograss and uniquely identify genotypes originating from the same geographic region (Budak et al., 2004a; Zhou et al., 2011).

SSR technology offers opportunities for buffalograss-specific molecular markers and can be developed from previous buffalograss sequence data. SSRs are codominant, multiallelic, and highly variable molecular markers that are distributed across organismal genomes (Jones et al., 2001). SSR molecular markers are generally between 100bp to 300bp long comprised of relatively short repeated motifs that range from two to six base pairs long. SSR polymorphisms are defined as variation in motif copy number and are likely caused by translation errors during DNA replication (Levinson and Gutman, 1987). SSRs have been used for genetic architecture assessment, DNA fingerprinting, line purity maintenance, marker assisted selection, and trait mapping in both warm- and cool-season turfgrasses (Budak et al., 2004a; Hadle et al., 2016; He et al., 2003; Jones et al., 2001; Saha et al., 2006). SSRs can be transferred to buffalograss from maize [*Zea mays* (L.)], sorghum [*Sorghum bicolor* (L.)], pearl millet [*Pennisetum americanum* (L.)], and sugarcane [*Saccharum officinarum* (L.)] relying on conserved primers flanking the SSR region (Budak et al., 2004a).

Genetic resources refer to the availability of genetic information and material for an organism. Common plant genetic resources include germplasm, seed repositories, the

National Center for Biotechnology Information (NCBI), and the Germplasm Resources Information Network (GRIN). Currently, genetic resources for buffalograss are limited. The University of Nebraska-Lincoln maintains a buffalograss collection of 2986 buffalograss accessions at Mead, Nebraska. Previously, Wachholtz et al. (2013) contributed to public buffalograss genetic resources by sequencing the transcriptomes of cultivars '378' and 'Prestige'. Transcriptome sequencing resulted in 506,719 total transcripts, and 325 differentially expressed transcripts between 'Prestige' and '378' when challenged with chinch bugs [*Blissus occiduus* (Barber)] (Wachholtz et al., 2013). Donze et al. (2015) sequenced the transcriptomes of ten male and ten female buffalograss F1 progeny from a genetic linkage mapping population and found 260,693 total transcripts, and 1734 differentially expressed transcripts (FDR < 0.01) by gender. Of those resulting transcripts, 445 had higher expression in male plants, and two were uniquely expressed in the males. In female buffalograsses one transcript was uniquely expressed, and 1289 transcripts had higher expression. Amaradasa and Amundsen (2016) sequenced twenty four buffalograss cDNA libraries from leaf spot resistant '95-55' and 'NE-BFG-7-3459-19' and leaf spot susceptible 'Prestige' and 'NE-BFG-73453-50', and mapped 98% of these transcripts to the 'Prestige' reference transcriptome at least once. Between leaf spot resistant and susceptible lines, ten differentially expressed genes were validated and the final reference was made available at NCBI BioProject Repository project ID PRJNA297834. These publicly available transcriptomes provide a wealth of sequence data and an opportunity to develop buffalograss-specific molecular markers and

could be a foundation for further marker assisted selection projects (Amaradasa and Amundsen, 2016).

High-throughput technologies such as high-throughput imaging and high resolution melt have been developed for rapid phenotype measurements in the field and genetic assessment using molecular markers in turfgrass (Walter et al., 2012; Warnke et al., 2016). High resolution melt analysis (HRM) is the analysis after PCR amplifies target DNA with DNA intercalating florescent dye. Florescence of the dye is recorded at the end of the extension phase of each cycle to quantify amplification during PCR, and after the final amplification cycle the florescence was recorded during the melting cycle to identify the temperature at which the amplified DNA molecule is completely denatured (Wu et al., 2009). The resulting melt curves from HRM can distinguish sequences with minor nucleotide differences (Figure 1.1), and by using a quantitative rtPCR system a relatively large number of samples or markers can be screened quickly (Simko, 2016). Previously, HRM was performed on poverty oatgrass [*Danthonia spicata* (L.)] (Thammina et al., 2016) and creeping bentgrass [*Agrostis stolonifera* (L.)] (Warnke et al., 2017) finding that markers can quickly and reliably be developed and screened at a low financial cost. HRM has not been previously used as a high-throughput screening method for buffalograss genotype discrimination.

Few species-derived markers have been developed for buffalograss and no methods for quickly screening PCR-based molecular markers have been tested in

buffalograss. Here, we describe the development of buffalograss-derived SSR markers using an in-silico analysis coupled with HRM to validate the SSR markers as a high-throughput screening method.

Materials and Methods

Plant material and DNA extraction. Buffalograss cultivars ‘315’, ‘378’, ‘Prestige’, ‘Legacy’, ‘609’, ‘95-55’, and ‘Density’ were grown in a greenhouse at the University of Nebraska, Lincoln. Plants were grown in four-inch containers in potting media consisting of 38% peat, 23% field soil, 19% sand, and 19% vermiculite. Plants were irrigated three times weekly, which was enough to prevent desiccation and maintain consistent plant growth. The greenhouse was maintained with supplemental lighting maintaining a 14-hour photoperiod with 23 °C - 27 °C day and 21 °C -23 °C night temperatures. Mowing was done with scissors as needed to maintain a 5 cm canopy height. Fresh plant tissue was collected by cutting juvenile tissue with a pair of scissors from the plant and added to a small labeled manila envelope. Once collected, plant tissue was flash frozen in liquid nitrogen. Genomic DNA was extracted from buffalograss using the Gentra Puregene Core Kit A (Qiagen, Valencia, CA) following the manufacturer directions.

Primer Design. Previously constructed transcriptome assemblies of ‘378’ and ‘Prestige’ buffalograss cultivars contained 95,501 and 91,519 respective contiguous sequences (Amaradasa and Amundsen, 2016). Simple sequence repeats were identified by mining the ‘Prestige’ transcriptome using the MIcroSAtellite identification tool (MiSA) (Coelle Coello and Cortés, 2005). MiSa identified mono-nucleotide repeats with at least ten copies, di-nucleotide repeats with six copies, and three through six base pair repeats with five perfect copies (Warnke et al., 2017). The same SSR identification process was repeated for the buffalograss ‘378’ transcriptome. The ‘378’ transcriptome was used as a

reference in a basic alignment search tool for nucleotide data (BLASTn) (Altschul et al., 1990) search, and the ‘Prestige’ transcriptome was used as the query. Conserved sequences were compared against MiSA output to identify conserved sequences between the tested transcriptomes that contained the same SSR. Conserved SSRs that differed in SSR copy number between genotypes were considered for further analysis.

PCR primers flanking the polymorphic SSRs were designed using Primer3 with default settings, ranging from 15 to 21 base pairs long. The target product sequence lengths were between 50 and 150 base pairs long. Primers were synthesized and purchased from Integrated DNA technologies (Coralville, IA) (Thammina et al., 2016; Warnke et al., 2016).

High Resolution Melt. HRM is a quick method to screen PCR-based markers and can be performed in 96- or 384-well plates and is relatively fast compared to other molecular marker detection formats (Warnke et al., 2017). HRM was performed and replicated two times with each DNA and primer combination. Each reaction had a total volume of 10µl per reaction in MPC-480W 96-well semi-skirted PCR plates (Phenix Research Products, Candler, NC) and the 96-well plates were covered with optically transparent film (LMT-RT2, Phenix research products). All PCR components were kept on ice during reaction preparation. The components for one reaction were 0.5µl both [$50nm^{-1}\cdot\mu l$] forward and [$50nm^{-1}\cdot\mu l$] reverse primers (Integrated DNA Technologies inc.), 0.5µl genomic DNA, 3.5µl ddH2O, and 5µl of DNA intercalating QuantiTect SYBR Green PCR Master Mix

(Cat No. 204145, Qiagen, Hilden, Germany). High resolution melt was performed on a Roche LightCycler480 (F. Hoffmann-La Roche AG, Basel, Switzerland) as follows, the heating block was initially heated to 95°C, followed by 50 cycles with denaturation temperature of 95°C, annealing temperature of 65°C, and extension temperature of 72°C. Each amplification cycle lasted 47.68 seconds, and during the extension phase of each cycle, fluorescence quantification was recorded. After 50 cycles, DNA melting started at 65°C and ramped up to 97°C at a rate of 0.11°C/s, and fluorescence quantification was recorded. Twenty-two markers that were conserved and polymorphic between '378' and 'Prestige' were screened on seven buffalograss cultivars, including '378' and 'Prestige' (Table 1.2).

Data Analysis. Simple sequence repeat markers were scored as dominant markers by melt temperature. Samples that denatured within one standard deviation from the mean melt temperature were scored a one. Samples that denatured outside of one standard deviation from the mean temperature were scored a zero. Samples that did not amplify were scored two for missing data. Each reaction was replicated two times and compared for consistency and fluorescence signal intensity.

A series of programs in the Numerical Taxonomy and Multivariate Analysis system version 2.21 (NTSYSpcC.2.21; Rohlf F.J, 2000) suite were used to process the data. Sørensen-Dice similarity coefficient of qualitative data (Simqual) was used to measure the similarity between differing genotypes based on the HRM data (Sørensen,

1948). An unweighted pair group method with arithmetic averages (UPGMA) dendrogram depicting genetic similarity based on shared markers was created using the sequential, agglomerative, hierarchical, and nested clustering method (SAHN) (Sneath and Sokal, 1973). Data was then normalized around zero using double centralized (Dcenter) data for principal coordinate analysis based on similarities of the data (Gowler, 1966). Using the Dcenter data, eigenvalue and eigenvector matrices were computed with Eigen, and produced principal coordinate analyses (PCA), showing similarities between genotypes in two dimensions.

Results and Discussion

Buffalograss derived SSR markers were developed and used to distinguish buffalograss cultivars. From the 95,501 total transcripts in the '378' transcriptome, MiSa identified 17,334 transcripts (18%) containing SSRs, consisting of 11,260 mono- (12%), 2,235 di- (2%) , 3,695 tri- (4%), 130 tetra- (.14%), and 14 penta-nucleotide (.015%) repeats. From the 91,519 'Prestige' transcripts, MiSa identified 14,223 (15%) total SSRs, consisting of 8,412 (9%) mono-, 2,003 di- (2%), 3694 tri- (4%), 103 tetra- (.11%), and 11 (.012%) penta-nucleotide repeats (Figure 1.2, Table 1.1). Lower complexity SSRs were the dominant classes consistent with other reports (Von Stackelberg et al., 2006). The proportion of SSR-containing transcripts is similar with other reports such as Rai et al. (2016) who found 26% of cell wall related genes from sorghum to contain SSRs. Similarly, Varshney et al. (2002) analyzed EST sequences from several cereal genomes and found the number of SSRs ranged from 7 to 10% of the analyzed sequences. Our results are consistent with previous reports when only dinucleotide and higher complexity repeats are considered, where SSRs were found in 6% of both the 'Prestige' and '378' transcriptomes.

A BLASTn search of the '378' transcriptome with the 'Prestige' transcriptome identified 65,830 (72%) conserved sequences. The relatively large number of conserved sequences was expected since '378' and 'Prestige' are both buffalograss cultivars. The differences are attributed to genotypic differences between the cultivars, incomplete transcriptome assemblies, and errors that were introduced during sequencing and

assembly that were not identified. A higher degree of confidence was placed in the conserved sequences since each transcriptome was assembled independently. Within these conserved sequences there were 259 polymorphic SSRs consisting of 155 di-, 102 tri-, and 2 tetra-nucleotide repeats (Figure 1.2, Table 1.1).

Primer3 software developed 139 PCR primer pairs from the conserved sequences, targeting polymorphic SSRs. A subset of 96 primer pairs were obtained and tested on ‘Prestige’ DNA, and 91 amplified. Twenty-two primer pairs were further tested on a panel of seven buffalograss cultivars, and all primers amplified with allele frequencies ranging from 0.29 to 1 in the tested buffalograss panel (Table 1.2). Polymorphic information content (PIC) values ranged from 0 to 0.916 for the markers tested. Of the markers tested, two (9%) had PIC values greater than 0.7 making them highly informative and four (18%) of the markers had PIC values between 0.4 and 0.7, making them moderately informative (Hildebrand et al., 1992) (Table 1.2). Markers with a greater number of alleles generally have greater PIC values, making them more informative (Hildebrand et al., 1992). Allele frequency of the markers ranged from 0.29 to 1. The markers were scored as dominant markers since some markers showed more than one alternate melt profile making it difficult to accurately score these markers as codominant.

High resolution melt proved to be a quick method to screen the twenty-two markers that were polymorphic between ‘378’ and ‘Prestige’ on seven buffalograss

cultivars, including ‘378’ and ‘Prestige’ (Table 1.2). Of the 22 markers, nine could discriminate varieties. A typical melt profile showing differences between the buffalograss cultivars ‘378’ and ‘Prestige’ is shown in Figure 1.1; the shift in melt temperatures reflects a polymorphism between the two samples (Figure 1.1).

The SSR markers were scored, and the resulting scoring matrix was used for UPGMA clustering that depicts genetic similarities (GS) of the samples tested. Genetic similarity ranged from 0.8 to 0.97 (Figure 1.3). The average genetic similarity coefficient between all samples tested was 0.9, the maximum similarity was 0.97 between ‘315’ and ‘378’, 0.93 between ‘Prestige’ and the ‘315’/’378’ cluster (Figure 1.3, Figure 1.4). These findings are consistent with other molecular marker studies where ‘315’, ‘378’, ‘Prestige’ and ‘609’ grouped together (Budak et al., 2004a; Budak et al., 2004b). The SSR marker E9 from table 2 was able to distinguish cultivars ‘378’ and ‘315’, contributing to the difference observed between cultivars (Figure 1.3, Figure 1.4). The lowest genetic similarities in the study were between the ‘315’/’378’ cluster and the ’95-55’/‘Density’ cluster (GS = 0.8). Further separation of cultivars may be achieved by a larger panel of markers. Budak et al. (2004a) achieved genetic similarity coefficients ranging from 0.04 to 1.0 using 330 SSR markers derived from other grass species. Molecular markers from other grass species can be adapted for use in buffalograss due to the high degree of synteny among grasses but may not offer enough discriminatory power to distinguish all cultivars as observed by Budak et al. (2004). Markers developed in this study could be added to a wider panel of markers for greater discriminatory power. Sheef et al. (2003)

reports that about 2% of SCAR markers developed from RAPD markers to be species specific in creeping bentgrass, potentially testing the full panel of 139 SSR markers developed would yield two or three buffalograss specific markers. However, when deriving markers from the study species, one could expect to find a larger proportion of species-specific markers. Adding not only a wider panel of makers, but a wider panel of buffalograss samples ($n \geq 25$) is necessary for accurate resolution of genetic diversity in cross pollinated populations of random plants (Wanke et al., 1997). The panel of SSR-based markers developed in this study are the first markers derived from buffalograss that can be used to genetically distinguish cultivars of buffalograss. HRM was used on these SSR markers to identify polymorphisms in the tested vegetative buffalograss cultivars. This study serves as a proof of concept that HRM can be performed on a panel of markers to distinguish buffalograsses. Further research should be performed on buffalograss to assess genetic diversity using a larger panel of buffalograss genotypes.

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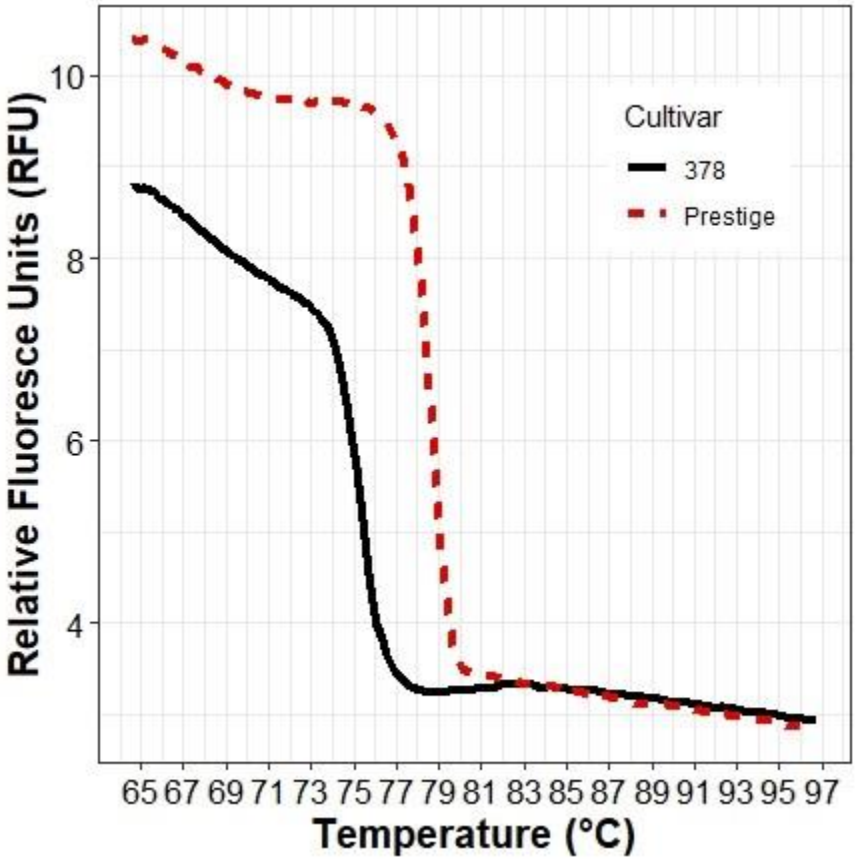

Figure 1.1 High Resolution Melt (HRM) profiles of '378' and 'Prestige' SSR amplification exhibiting a polymorphism

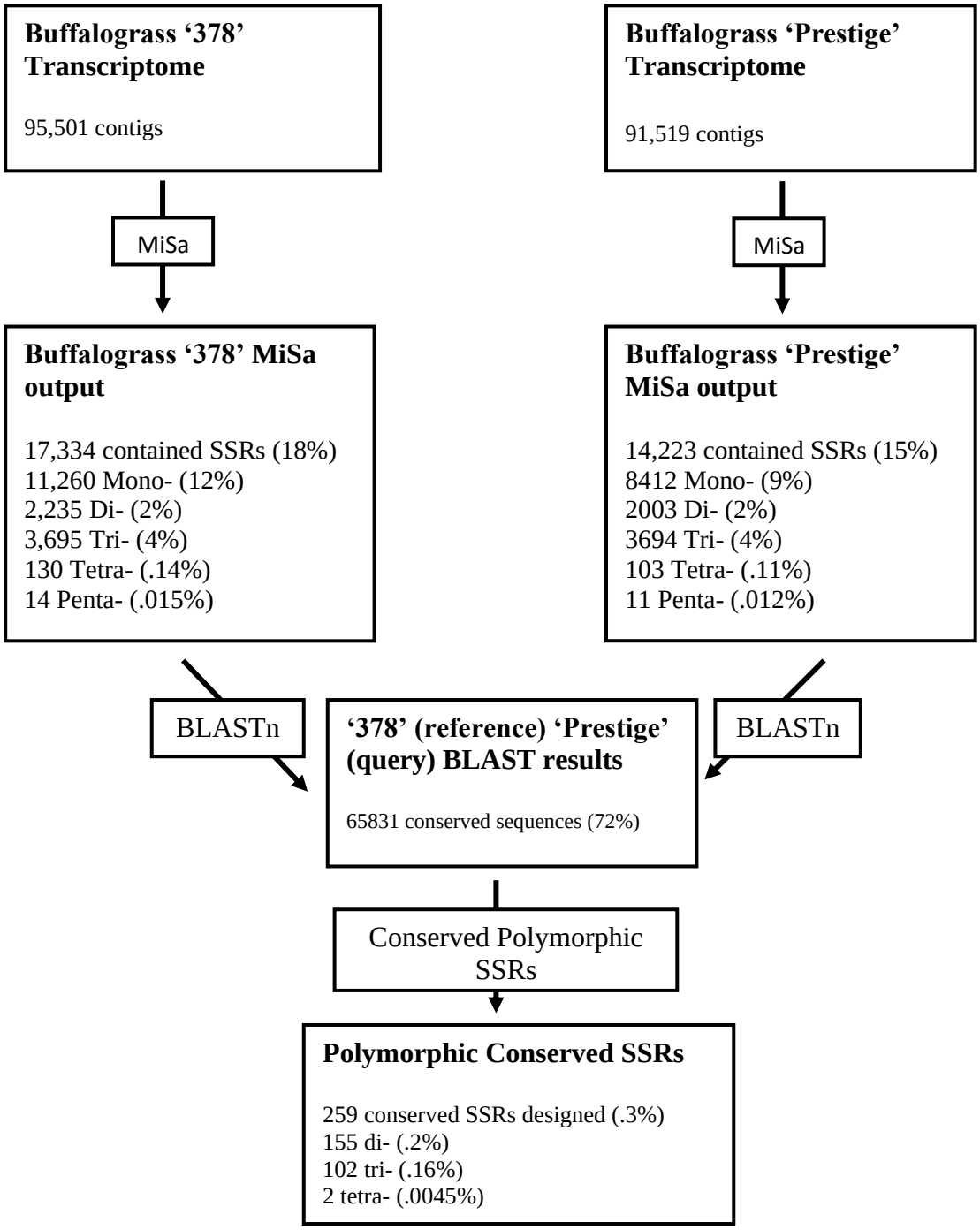

Figure 1.2 SSR development pipeline starting from raw transcriptome input files (FASTA format) followed by separately performing MiSa on each transcriptome. A BLASTn search was performed using '378' as a reference and 'Prestige' as a query. Conserved polymorphic SSRs were identified by comparing the MiSA and BLASTn outputs.

Table 1.1 Buffalograss ‘378’ and ‘Prestige’ MiSa SSR identification summary. Identified SSR motifs between ‘378’ and ‘Prestige’ transcriptomes were compared for conserved and polymorphic SSRs

Cultivar	Identified SSRs	Monomer	Dimer	Trimer	Tetramer	Pentamer
‘378’	17334	11260	2235	3695	130	14
‘Prestige’	14223	8412	2003	3694	103	11
Conserved SSRs between transcriptomes	53270	-	155	102	2	0

Table 1.2 Frequency of '378' and 'Prestige' SSR amplification on a panel of seven buffalograss cultivars. SSRs are described by the motif and copy number. Amplification frequency was assessed across seven buffalograss cultivars.

Primer Name	Primer Pair Sequence	SSR Motif	'378' Copy Number	'Prestige' Copy Number	Amplification Frequency	(PIC) Value
A4	5' CTGGCGTCGACCCACATG 3' 5' GAGCTCGACCCACGTCAC 3'	"TC"	6	8	1	0
A10	5' TCGGCCTGACATTAGTTTGAA 3' 5' CTGGCACCAGCATAAATGGC 3'	"ACC"	6	5	.57	0.675
B4	5' CCCCAACTCAGGTCAGTGC 3' 5' TCTGCAGTGTGAGAGGGGA 3'	"GTA"	7	5	.57	0.675
B9	5' CACCGACCGGCCGATC 3' 5' CCCAGAACCAGGGGTGGA 3'	"GCA"	6	5	1	0
B11	5' CGCATTTTCTCCGGCAGC 3' 5' CGACTCCCAAAACCAGAGCA 3'	"TTC"	5	7	1	0
C3	5' ATCCCTCCCCTGCCCATT 3' 5' TTCTGTTTCGGCGTCGGG 3'	"CT"	8	9	1	0
C4	5' CCCTCCCTCTCCCTTGCT 3' 5' TTGCTACGCTTCCCCACG 3'	"CG"	8	9	1	0
C7	5' TGAGGGGCGAAGAGACGA 3' 5' TGGATTGGATTGGAACCAGCT 3'	"GA"	10	6	1	0
C10	5' AGGACGCGAATGACGACG 3' 5' GGCTTCAGGTCCCGGAAC 3'	"GAG"	6	5	1	0
C12	5' CCACTCCAAAGCGCCTGA 3' 5' CTCCTTGGGGCCGAACAG 3'	"GGA"	5	6	.71	0.496
D1	5' TGTTGGAGTGATCTCAGGTGC 3' 5' GGCCAATCTGCAACCCCT 3'	"CT"	7	8	.29	0.916
D2	5' CACAGAGGCATTCCCCGG 3' 5' TTGCACTGCTCCCTCAGG 3'	"TG"	7	9	.71	0
D10	5' TGCTTCTGGCGATCCGAC 3' 5' AGAACAAGGAGCAGGCGT 3'	"ATCT	5	6	1	0
E6	5' TTTGGTGCGGAGAGGACG 3' 5' TCCACCCACCTTCCCCTC 3'	"GA"	10	8	1	0
E8	5' TCTAAGGTTGGGCGCTCC 3' 5' GTATCGCCCTCGCCTTCG 3'	"CGC"	7	5	1	0
E9	5' GCGCTCGTGCCTTTCAAC 3' 5' TATGACCCGCGCGAACAG 3'	"TC"	8	10	.85	0.278
F1	5' GATGCCCCGCTCCTCG 3' 5' TGCATCAGCGTCCCCTTC 3'	"GGC"	6	5	.85	0.278
F3	5' TCCGACGTGCTGGGTTTG 3' 5' TCCGACGTGCTGGGTTTG 3'	"TA"	7	8	.57	0.675
G1	5' CCCAGCACCCAACGATCA 3' 5' CACCCCGTCACCCGAAG 3'	"TC"	11	6	.43	0.815
G10	5' CTCCAGGCCGCCAAGAAA 3' 5' TACCCGTCCACCAGTCGT 3'	"GCG"	5	6	1	0
G11	5' CTCCAGGCCGCCAAGAAA 3' 5' TACCCGTCCACCAGTCGT 3'	"GCC"	5	6	1	0
H12	5' AACAGCACCAGTTCCGCT 3' 5' CCTTGGTGCGACTGAGCA 3'	"AGA"	5	7	1	0

¶ PIC value represents polymorphism information content value

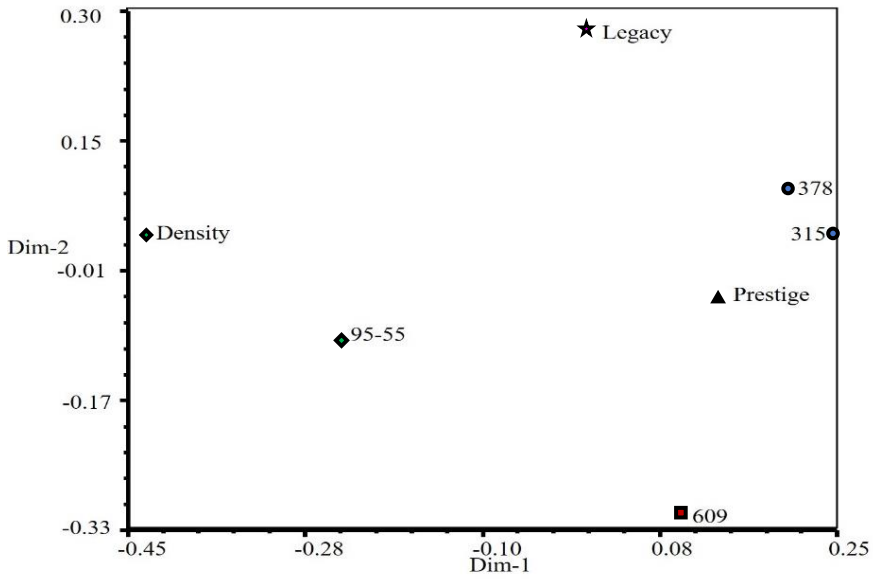

Figure 1.3 Principal coordinate analysis, depicting genetic differences with the greatest degree of similarity along the x-axis (Dim-1), and smaller genetic differences along the y-axis (Dim-2).

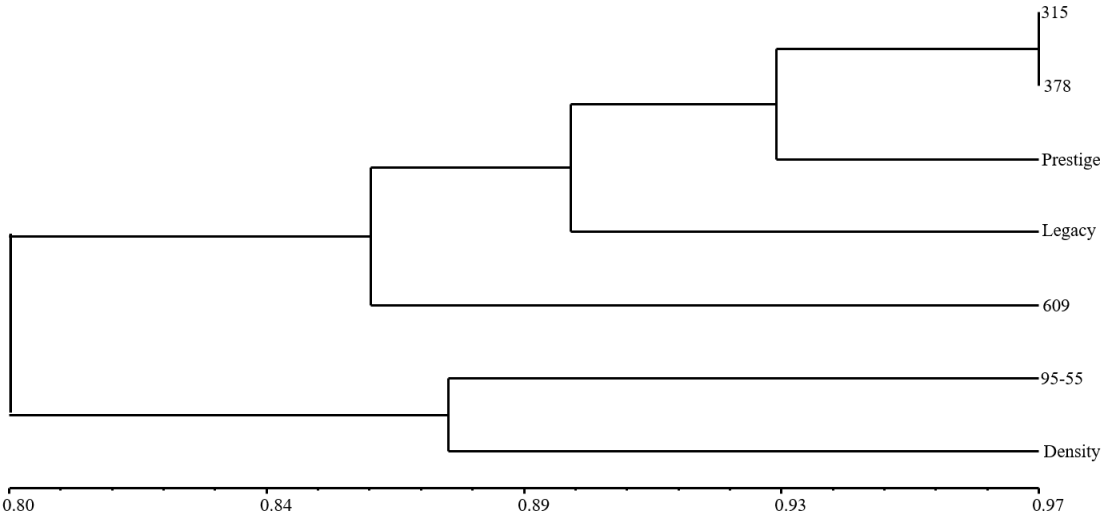

Figure 1.4 Unweighted pair group method arithmetic averages (UPGMA) show genetic similarity of cultivar clustering. Similarity coefficient is depicted along the x-axis, and sample name is displayed along the y-axis

CHAPTER TWO

DIVERSITY ASSESSMENT OF WILD AND ELITE BUFFALOGRASS GENOTYPES

Abstract

Buffalograss is a low input, stoloniferous, warm season turfgrass species native to the central Great Plains of North America. These characteristics make it a valuable turf for the region. Buffalograss is a naturally diverse species due to its obligate outcrossing nature. The objectives of this study were to collect and assess diversity of wild and elite buffalograss accessions using recently developed buffalograss derived simple sequence (SSRs) markers, phenotypic traits, and flow cytometry. Fifty-eight collected buffalograss genotypes were collected from Nebraska, Kansas, Oklahoma, Texas, New Mexico, and Colorado, and established at the University of Nebraska Lincoln's turf research farm and research greenhouse. Using the molecular marker data; phylogenetic relationships were drawn using SAHN and EIGEN programs in NTSYSpcC.2.21. Variation of turfgrass performance traits was associated with the geographic location of collection sites. Unweighted pair-group arithmetic mean clustering found genetic similarity coefficients for the collection ranging from 0.46 to 1.0, and the average genetic similarity was 0.82. Phenotypic differences were detected between genotypes, and between collection sites. The new buffalograss genotypes collected in this study may benefit buffalograss breeders and turf managers and lead to the development of high-quality low input buffalograss cultivars.

Introduction

Buffalograss [*Buchloë dactyloides* (Nutt.) Engelm.] syn. [*Bouteloua dactyloides* (Nutt.) Columbus] (Columbus J.T., 1999) is a low input, warm-season grass species, and is native to the United States Great Plains, distributed from Mexico to Canada, along the eastward foothills of the Rocky Mountains and into eastern Nebraska, Kansas, Oklahoma, and Texas (Shearman et al., 2004). Buffalograss is valuable as a turfgrass because it requires low amounts of irrigation, fertility, and mowing, while maintaining acceptable visual quality (Goss, 2017; Johnson et al. 2000; Frank et al. 2002; Morris, 2013; Steinke et al. 2011). However, further research and improvement is needed for buffalograss to be competitive in visual quality when compared to commonly used turfgrasses such as Kentucky bluegrass [*Poa pratensis* (L.)], creeping bentgrass [*Agrostis stolonifera* (L.)], or tall fescue [*Lolium arundinaceum* (Schreb.)]. Buffalograss presents unique opportunities for cultivar development and wide adoption as a natural alternative turfgrass in the Great Plains region.

Buffalograss is an obligate outcrossing dioecious turfgrass species which contributes to wide genetic variation and has potentially valuable traits than can be improved through selective breeding. (Browning et al., 1994; Huff et al. 1993). Natural selection pressures have been imposed on wild buffalograss throughout the habitat and diverse buffalograss ecotypes have adapted to their environments in response (Browning et al., 1994). Wild buffalograss stands offer the widest range of unimproved traits. Collecting from natural or minimally maintained buffalograss stands present an

opportunity to expand breeding genetics by adding diverse buffalograss genotypes to collections. One way to study the underlying genetic diversity in plants is to use molecular markers.

Buffalograss diversity has been assessed with several different molecular markers; these markers used to study buffalograss diversity include, Inter-simple sequence repeat (ISSRs), randomly amplified polymorphic DNA (RAPDs), restriction fragment length polymorphism (RFLP), sequence related amplified polymorphism (SRAPs), and simple sequence repeats (SSRs) (Budak et al., 2004a; Budak et al., 2004b; Huff et al., 1993; Wu and Lin, 1994; Zhou et al., 2011). ISSRs produced 207 polymorphic bands from 30 ISSR markers. ISSRs consistently separated genotypes of buffalograss, revealing high levels of genetic distances between cultivars with an average similarity of 0.51. RAPD markers had the least distinguishing power but in RAPD analysis buffalograsses from similar geographic regions generally grouped together. There were 249 polymorphic bands from thirty SRAP markers. SRAPs strongly differentiated vegetative genotypes from seeded genotypes (Budak et al., 2004a, Budak et al., 2005). When SRAPs were compared with ploidy, genotypes were grouped by ploidy. RFLPs tested on buffalograss had high rates of polymorphism, with 189 polymorphic bands out of 225 bands present (Gulsen et al., 2005a). SSRs tested on buffalograss were derived from maize, pearl millet, sorghum, and sugarcane. SSRs had a high rate of polymorphism (87%), and revealed high genetic distances (Budak et al., 2004a). Buffalograss diversity was inspected by Hadle et al. (2016) across 14 loci by

implementing buffalograss derived SSRs. On average 19 alleles were identified per tested SSR ranging in size from 115bp – 406bp long. Average heterozygosity of the markers was 0.86, which is notably higher than other outcrossing species previously observed (Hadle, 2016; Nybom, 2004). In a combined analysis with these markers, elite genotypes of buffalograss have been assessed, finding high average similarities and unique genetic fingerprints. Molecular markers can also be correlated with ploidy level in ploidy series.

Hadle et al. (2019) estimated ploidy of 364 herbarium or field collected buffalograss genotypes using 14 buffalograss SSRs identifying multiple alleles at a locus, finding that SSR determination was an efficient method of ploidy determination and allele copy number had a linear relationship with ploidy. Limited information is available about wild buffalograss genotypic and phenotypic diversity. In effort to expand knowledge of buffalograss diversity and available genetics of the species, wild and elite genotypes should be assessed using markers developed in the previous study, in tandem with phenotypic buffalograss trait assessment.

Buffalograss has a diploid chromosome count of 2n = 20 (Reeder, 1971). Since then, flow cytometry has been used in buffalograss to study ploidy, revealing diploid, tetraploid, pentaploid, and hexaploid populations (Johnson et al., 1998, Johnson et al., 2001). Studies exploring the distribution of buffalograss have been conducted to determine ploidy in wild southern populations, finding four ploidy levels are found throughout the Great Plains region. Diploid populations are more southerly distributed

and the least common ploidy level. Tetraploid populations were westerly adapted, hexaploid populations are found throughout the habitat and are the dominant ploidy level representing 73% of the genotypes tested (Budak, et al., 2005 Johnson et al., 1998; Johnson et al., 2001). Johnson et al. (2001) notes a positive relationship between higher ploidy levels and cold tolerance in buffalograss.

Buffalograss is adapted to a wide range of habitats and environmental stresses including drought, heat, and cold tolerance. Phenotypic traits important for buffalograss used for turf include seed yield, establishment rate, fall color, genetic color, spring green up, visual and functional quality, low fertility requirements, canopy density, pest resistance, low mowing tolerance, vertical elongation rate, and drought, heat and cold tolerance (Browning et al., 1994; Frank et al., 2000; Frank et al., 2002; Frank et al., 2004; Goss, 2017; Heng-moss et al., 2002; Johnson et al., 1997; Kenworthy et al., 1999). Wide buffalograss phenotypic variation for quality and color has observed in natural buffalograss stands. Heritability of visual quality, color, seasonal color, vertical elongation, and stolon characteristics have been measured resulting in color and vertical elongation being highly heritable (Browning et al., 1994). Phenotypic traits and genetic sequences can be selected for using marker assisted selection (MAS) (Budak et al., 2004a; Gulsen et al., 2005b). Vogel et al., (2018) mapped big bluestem (*Andropogon gerardii* Vitman) and indiangrass (*Sorghastum nutans* Nash) by geographic origin and investigated biomass by geographic origin and observed differences in biomass production per plant by north or south location of collection site in both species.

Evidence of varying traits on a north to south spectrum were observed in wild buffalograss for winter dormancy and drought tolerance (Johnson et al., 2001; Kenworthy et al., 1999). Since buffalograss ploidy and winter dormancy are associated with geographic distribution it is important to consider geographic location of the entries when assessing buffalograss phenotypic traits. Buffalograss is a dominant grass species from Central Mexico to Canada, growing from the eastern foothills of the Rocky Mountains and into eastern Nebraska, Kansas, Oklahoma, and Texas. From this region, wild buffalograss should be collected from minimally maintained landscapes in order to introduce wild and varying genetics of buffalograss into the University of Nebraska buffalograss breeding program. A collection of new buffalograss genotypes presents an opportunity to introduce new and potentially valuable genetics to a breeding population. Evidence of varying traits on a north to south spectrum were observed in wild buffalograss for fall dormancy and drought tolerance (Johnson et al., 2001; Kenworth et al., 1999).

Wild buffalograss diversity has not been assessed by combining buffalograss derived markers, flow cytometry, phenotypic traits and geographic mapping of the collection site. Together, these methods can provide a better understanding of species diversity. The primary goals of this study were to assess genetic similarity and evaluated phenotypic traits of a minimally maintained collection and elite buffalograss genotypes. The goal was achieved by 1) collecting wild buffalograss accessions and expanding the University of Nebraska buffalograss collection, 2) assessing genetic relationships

between collected and elite buffalograss genotypes using buffalograss-derived SSR markers, 3) comparing lateral spread, stolon count, stolon width, genetic color, fall color, spring color, and visual quality of the collection, elite breeding material, and select cultivars. Resulting information from these objectives can be used by plant breeders for cultivar development in the interest of creating improved buffalograss cultivars. A collection of buffalograss from minimally maintained locations, may add underrepresented genetics to the buffalograss breeding program at the University of Nebraska at Lincoln.

Materials and methods

Fifty-eight wild buffalograss accessions were collected from minimally maintained landscapes in Nebraska, Kansas, Oklahoma, Texas, New Mexico, and Colorado. Samples were collected using a trowel and stored in a zip top bag with a moist paper towel. Samples were labeled, and date and location (GPS derived longitude and latitude) of each were recorded. The newly collected buffalograss was overnight shipped to the University of Nebraska (Lincoln, NE) and established with select elite breeding material in a greenhouse with supplemental lighting from six to eight AM and five to eight PM into four-inch square pots containing 19% vermiculite, 19% sand, 38% peat, and 23% field soil. Plants were irrigated as needed and trimmed with scissors to prevent contamination from neighboring plants. Leaf tissue that was two weeks old or less was collected with scissors and flash frozen in liquid nitrogen. Genomic DNA was extracted from frozen

samples using the Gentra Puregene Core Kit A (Qiagen, USA inc, 27220 Turnberry Lane, Valencia, CA 91335).

High resolution melt PCR was performed and replicated two to three times with each DNA and primer combination. Each reaction had a total volume of 10µl per reaction in MPC-480W 96-well semi-skirted PCR plates (Phenix Research Products, Candler, NC) and the 96-well plates were covered with optically transparent film (LMT-RT2, Phenix research products, Candler, NC). All PCR reaction components were kept on ice during reaction preparation. Each reaction consisted of 0.5µl of [$50ng^{-1}\cdot\mu l$] forward and 0.5 µl [$50ng^{-1}\cdot\mu l$] reverse primers, 0.5µl template DNA, 3.5µl nuclease free H_2O, and 5µl of DNA intercalating QuantiTect SYBR Green PCR Master Mix (Cat No. 204145, Quigen, Hilden, Germany). High resolution melt (HRM) was performed on a Roche lightcycler480 (F. Hoffmann-La Roche AG, Basel, Switzerland) as follows. The heating block was initially heated to 95°C, followed by 50 cycles with denaturation at 95°C for 10 seconds, annealing at 65°C for 10 seconds, and elongation at 72°C for 10 seconds; fluorescence quantification was taken during the elongation phase. After 50 cycles, the DNA melting phase started at 65°C and the temperature ramped up to 97 °C at a rate of 0.11°C/s. The fluorescence was recorded during this melting phase from 65°C to 97°C.

The simple sequence markers developed from the previous study were scored as dominant markers by melt temperature. Samples that denatured within one standard deviation from the mean melt temperature were scored a one. Samples that denatured

outside of one standard deviation from the mean temperature were scored a zero. Samples that did not amplify were scored two for missing data. Each DNA was replicated two times, and the sample that had the clearest amplification signal was scored.

Numerical Taxonomy and Multivariate Analysis system version 2.21 (NTSYSpcC.2.21) (Rohlf F.J, 1998) was used for genetic analysis. A Sørensen-Dice similarity coefficient of qualitative data (Simqual) was used to measure the similarity between genotypes. An unweighted pair group method with arithmetic averages (UPGMA) dendrogram depicting genetic similarity based on shared markers was created using the sequential, agglomerative, hierarchical, and nested clustering methods (SAHN) (Sneath and Sokal, 1973). Using double centralized (Dcenter) data and EIGEN, eigenvalue matrices were computed to produce principal coordinate analyses (PCA), showing similarities between genotypes in two dimensions (Gowler, 1966).

During phenotypic evaluations, collected buffalograss accessions and named buffalograss cultivars were arranged in a randomized complete block design with three replications at the University of Nebraska, Lincoln turfgrass research farm (40°50'10.9"N, 96°39'54.5"W) in May of 2018. Phenotypic traits were evaluated from May 2018 to June 2019 (Table 2.1). Phenotypic traits that could be discretely and quantitatively measured were lateral spread, stolon count, and stolon width. Lateral spread was measured across each entry measuring to the nearest centimeter using a meter stick. Stolons of each entry in the evaluation that had three or more nodes were counted. Stolon internode width

proximal to the third node were measured in micrometers (μm) using a caliper. other henotypic traits were subjectively evaluated on a visual 1-9 scale following the National Turfgrass Evaluation Program guidelines. Phenotype data were collected from May to November 2018, and May to July 2019. The traits visually scored were genetic color, fall color, spring color, and quality. Color ratings of a one represents entries that were mostly brown or tan, whereas ratings of nine represent entries with a rich green color. Visual quality was rated with one representing poor overall uniformity, poor lateral spread, sparse canopy, and being brown or tan. Plots scoring a nine had rich green color, uniformity, good lateral spread, and a dense canopy. To test for differences in means between genotypes, analysis of variance was performed at a significance level of 0.05 (Littell et al., 20016). Fisher's LSD was used to separate means of evaluated genotypes at $P < 0.05$. Statistical analysis was performed in R (version 3.6.3). Significance of these traits were tested at a p-value of 0.05, and for all traits evaluated. Only evaluations with the highest significance are reported within the study. These evaluation procedures determine if the newly collected entries perform as well or better than named cultivars for these evaluated traits. These new buffalograss entries may be integrated into the university's buffalograss collection and may contribute valuable traits to new buffalograss cultivars in development. Phenotypic traits were evaluated for relationship to geographic location of collection site according to Vogel et al., (2018). Samples were divided by location north and south of 35.7°N. Samples were also divided by location east and west of 102°W. This dissected the collection region into an xy-plane with the origin at 35.7°N, 102°W. For subsequent analysis, the samples were pooled and averaged

in respect to the x or y axis. Analysis of variance and Fisher's LSD were performed examining location by trait of interest.

Ploidy determination and flow cytometry. Flow cytometry was performed on the collection. For each sample, approximately 0.5cm^2 of leaf tissue was chopped using a double-edged razor blade in 500µl chopping buffer until homogenous and allowed to incubate at room temperature for one minute. Each sample was then passed through a cell strainer cap 5ml test tube (#0877123, Fisher Scientific). To each sample, 2ml of propidium iodide was added as the staining solution. Prepped samples were kept on ice in the dark before analysis. The manufacturer's protocol for CyStain PI Absolute P nuclei isolation kit (#05-5022, Sysmex, Partec GmbH, Am Flugplatz 13, 02828, Gorlitz, Germany) was followed. Prepped samples were run on a Beckmen Coulter CytoFlex LX cytometer at the Flow Cytometry lab, University of Nebraska-Lincoln. The BluFl2-A channel was used for florescence measurement with a 575nm/30mW laser. Ploidy levels were calculated by $Chr_s = MDG_{ls} * Chr_r/MDG_{ls}$, where Chr_s represents the chromosome number of the sample, MDG_{ls} represents the median DNA content of the sample, Chr_r represents the chromosome number of the reference sample, and MDG_{lr} is the median DNA content of 'Density' buffalograss, a diploid.

DNA content was calculated by $DCS_{pg} = MDG_{ls} * MDG_{lp}/2c$, where DCS_{pg} represents DNA content of the sample in picograms, MDG_{ls} represents the median DNA content of the sample, MDG_{lp} represents the median DNA content of the reference

sample, and $2C$ is the DNA content 0.93pg/nucleus of ‘Density’ (Dirihan et al., 2013; Riordan et al.,2000).

Results and Discussion

Simple sequence repeat markers previously developed (see Chap 1) were implemented to assess diversity of newly collected and elite buffalograss genotypes and select buffalograss cultivars. The panel of 24 simple sequence repeat markers were scored as dominant markers and compiled into a similarity matrix using NTSYSpcC.2.21. Genetic similarity coefficient of the 98 evaluated buffalograss lines ranged from 0.46 to 1.00 (Figure 2.1). The average genetic similarity was 0.82, the maximum similarity was 1.00 and was between ’81’ and ’15-3693’ and between ‘48’ and ‘11-3612’, the minimum genetic similarity was 0.46 between ’09-3454’, ‘6’ and the rest of the collection (Figure 2.1). Using a panel of 330 SSRs on a panel of 15 elite buffalograss breeding lines and buffalograss cultivars, Budak et al. (2004a) found that buffalograss ‘Prestige’ and ‘609’ had a 0.89 genetic similarity, whereas ‘315’ and ‘378’ had a 0.84 genetic similarity. Using SSR markers derived from other grasses, Budak et al.(2004a) observed genetic similarity coefficient ranging from 0.04 - 1.00. The markers tested in this study expand buffalograss genetic resources and can be added for use in germplasm maintenance, purity, and genetic discrimination. The principal coordinate analysis (PCA) shows two major outlier samples, and one of these buffalograsses, ‘09-3454’, is from the University of Nebraska’s buffalograss mating populations and was the least similar to the rest of the population. The other outlier sample was entry ‘6’ which was harvested geographically near ‘7’ which grouped with the rest of the population (Figure 2.2). Wild blue grama used as an outgroup were intermixed with the buffalograsses with no clear distinction between the two species (Figure 2.1, Figure 2.2). This phenomenon may be attributed to the

synteny and close relation of the two grass species (Columbus, 1999.) Using a larger panel of SSR markers could further differentiate the two genotype groupings that were not distinguishable, create a larger range of genetic similarity, and add clarity to germplasm purity and maintenance.

Using flow cytometry to distinguish ploidy of the new collection by means of observing nuclear DNA content has been performed previously and is important for understanding the diversity of available genetic material (Budak et al 2005; Johnson et al., 1998; Johnson et al., 2001). Four percent of the entries collected were diploid with an average genome size of 0.83 pg DNA/nucleus (Figure 2.3). Collected diploid entries were found in the central to northern collection area, in contrast to previous reports indicating that diploids are from southern regions (Figure 2.4, Figure 2.5). This finding may be attributed to the relatively few diploids collected in this study ($n < 30$). Seven percent of the samples were tetraploid with an average DNA content of 2.05 pg DNA/nucleus (Figure 2.3). Tetraploid entries were westerly located throughout the collection area and was found throughout the north to south range(Figure 2.4, Figure 2.5). Forty-eight percent of the samples examined were pentaploid averaging 2.87pg DNA/nucleus, and thirty-nine percent of the samples were found to be hexaploid averaging 3.7pg DNA/nucleus (Figure 2.3). Ratios of cytotypes collected in this study, specifically tetraploid and pentaploid representation are not consistent with previous studies (Hadle et al., 2019; Johnson et al., 1998; Johnson et al., 2001). Both pentaploids and hexaploids were found evenly distributed throughout the collection region (Figure 2.4, Figure 2.5).

Ploidy determination of cultivars ‘315’, ‘378’, ‘Density’, ‘Sundancer’, and ’95-55’, were pentaploid, hexaploid, diploid, hexaploid, and hexaploid respectively and were consistent with previous reports (Budak et al. 2005; Johnson et al. 1998). Positive relationships have been observed comparing buffalograss ploidy to phenotypic traits such as root tensile strength and cold tolerance (Giese et al., 1997). Higher ploidy levels also correlate with cooler temperature and geographic location (Hadle et al., 2019).

Phenotypic traits were compared to geographic locations of collected genotypes. Vogel et al. (2018) found that big bluestem [*Andropogon geradii* (Vitman)] and Indian grass [*Sorghastum nutans* (Nash)] varied in biomass production per plant by location. When plotted on a map in relation to origin, biomass was found to be significant on north to south and east to west axes for both species, but there were no significant differences on diagonal axes from the point of collection to the point of evaluation (Vogel et al., 2018). Geographic mapping maybe a valuable tool aiding in locating and assessing buffalograss with valuable traits.

Lateral distance measured in inches across actively growing tissues of each of the 58 newly collected buffalograss entries over time showed differences based on genotype and rating date (Table 2.2). This result showed that there are differences from one genotype to another in lateral distance across the plot at one time point. There is strong evidence that lateral distance of buffalograss changes based on time and method of establishment. Establishment has been tested through ‘percent cover’ between elite

genotypes and showed significant variation between lines (Riordan et al., 1993). When including geographic location data, geographic longitudes of where the buffalograsses were collected had pronounced relationship with the lateral distance of the accession (Table 2.3, Figure 2.6, Figure 2.7). Interestingly, there was an interaction between the lateral spread of samples and longitude origin (Table 2.3). Easterly adapted genotypes had a wider lateral spread, likely due to addition to higher precipitation at entries' collection origin. When examined geographically, buffalograss started showing differences in lateral spread by five weeks after planting. A wide variation of lateral spread was observed by the collection and Fishers LSD identified six confidence interval groupings within the evaluated collection (Table 2.4). The wide range of lateral spread represented by this collection and the buffalograss species offers possibilities for improving establishment rate, since the rate at which a genotype spreads is related to establishment rate (Riordan et al., 1993).

There was a positive relationship observed between lateral spread and stolon count. Analysis of variance was performed on this new buffalograss collection for stolon count and width data showed differences between samples (Table 2.5, Table 2.6). Browning et al. (1994) found stolon count and width to be heritable traits through maternal lines. Differences were observed for stolon count and stolon width within this collection, Maternal back-cross breeding may be an important method of integrating these traits for buffalograss cultivar development. Differences were identified between samples collected East of 102°W and West of 102°W (Table 2.7). Two, four, six, and

seven weeks after planting there was an interaction between the North-South axis and East-West axis in stolon count in samples collected from the northwestern quadrant in Colorado to the south eastern quadrant in Texas (Table 2.7, Figure 2.8, Figure 2.10). The samples were grouped into two confidence intervals (Table 2.8). Analysis of variance testing stolon width showed differences within the collection (Table 2.8), and further inspection shows no difference based on geographic location (Table 2.8). Fisher's LSD identified three confidence interval groupings within the 58 evaluated entries. (Table 2.9). Stolon width and lateral distance had a positive relationship, but there is limited literature comparing these measurements.

Previously mentioned evaluated traits all contribute to uniform turf with a dense canopy and high visual quality. Visual quality was rated on a 1-9 scale based on the uniformity, color, and canopy density. Analysis of variance testing for visual quality of each entry showed differences between genotypes (Table 2.10), and differences between latitude of collection site (Table 2.11). Fishers LSD based on latitude revealed differences between the northern half of the collection region, and the southern half (Table 2.11). A reason for increased visual quality between the regions, may be the proportion of ploidy groups collected. Entries from the north western quadrant had a higher concentration of hexaploid occurrences, compared to buffalograsses collected from the southwest and northeast where ploidy tended to be more evenly distributed (Figure 2.5, Table 2.12). Abeyo and Shearman, (2009) report that variation in quality and other performance characteristics of unimproved lines is greater than in advanced lines. Observed variation

in quality suggests that the new wild accessions could be used as a source to improve buffalograss quality through plant breeding. Browning et al., (1994) found phenotypic variance of quality in female buffalograss lines to be 1.97, rated on a 1 to 9 scale, in August of 1989. Heritability estimates ranged from 0.14 ± 0.04 to 0.36 ± 0.16 for August quality are promising for this trait in potential cultivar development (Browning et al., 1994). Since visual quality is a heritable trait, the variation in quality and other traits observed within this collection offer valuable genetics, that can potentially contribute to cultivar improvement.

Highly rated fall color contributes to fall visual quality of buffalograss. Analysis of variance of fall color showed differences between entries, between collection dates, and there was an interaction between sample and date (Table 2.13, Table 2.14). All collection dates showed differences between samples, over three weeks (10/26/2018 - 11/16/2018) the mean visual color ratings fell from 3.6 to 0.98 (Table 2.14). This change in color represented a moderately green stand transitioning to a completely dormant buffalograss stand. Observed differences between collection dates are expected when the winter dormancy response is initiated. Results from this study are consistent with Kenworthy et al., (1999) findings that the onset of fall color began in late October and color ratings dropped rapidly over the following month into dormancy. In the 1999 study only two of the 280 genotypes had dropped below a visual rating of four, into the following month Kenworth notes that 252 other genotypes scored below a four in fall color after numerous frosts. If the accessions do not go dormant with increasing intensity

of cold, then they would likely not be winter hardy and would die. Pooling the samples into quadrants showed that there were differences between longitudinal hemispheres (Table 2.15). Based on Fisher's LSD, entries collected west of 102°W possessed a stronger dormancy response than entries collected east of 102°W (Table 2.16). Fishers LSD groupings of the collection separated the entries into two groupings (Table 2.17).

The collection showed differences in spring color for collection dates 4/19/2019 and 4/25/2019 (Table 2.18 , Table 2.19). Spring color was rated on a 1-9 scale with a score of one representing a brown dormant entry, and a rating of nine representing a rich green plot. The highest visual spring color rating was three and the lowest visual color rating was one. Kenworthy et al. (1999) report spring color evaluations from Lubbock, TX with visual ratings of 3.5 to 6.5, consistent with this study if considering the range to be shifted towards lower visual color ratings because of the later onset of warmer temperatures. Fisher's LSD grouping showed three groups for spring color on 4/25/2019 (Table 2.20). When pooled by geographic location, entries displayed differences based on lateral hemisphere of collection (Table 2.21), with northerly adapted buffalograsses exhibiting greater visual color ratings at time of evaluation (Table 2.22). In this study, northerly adapted buffalograsses exhibit higher visual color ratings and although there were less variation in spring color compared to fall color, both traits are promising aspects of cultivar development in comparison to Abeyo and Shearman (2009).

The results of this study demonstrate that these newly collected buffalograsses offer valuable additions of diverse genetics for the breeding program at the University of

Nebraska-Lincoln. Phenotypic traits of buffalograss found to be heritable in previous studies were related to geographic location of collection site. Based on the tested SSR markers, the University of Nebraska breeding program is maintaining genetically diverse germplasm. Further investigation using a larger panel of buffalograss derived markers would provide more genetic information to better characterize genetic diversity and resolve relationships between entries. Geographic mapping of the entries and assessing based on marker data should be applied to this new collection to investigate genetic trends in relationship to geographic location.

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Table 2.1. Phenotypic traits and the evaluation dates of fifty-eight buffalograss accessions evaluated in Lincoln, Nebraska.

Trait Evaluated	Dates Evaluated					
Lateral Distance	6/28/2018	7/12/2018	7/19/2018	7/24/2018		
Stolon Count	6/12/2018	6/28/2018	7/7/2018	7/10/2018	7/12/2018	7/19/2018
Stolon Width	8/2/2018					
Visual Quality	6/5/2018	6/7/2018	6/12/2018	6/28/2018	6/24/2019	
FallColor	10/19/2018	10/26/2018	11/2/2018	11/16/2018		
Spring Color	4/19/2019	4/25/2019	5/10/2019	5/17/2019	5/22/2019	5/31/2019

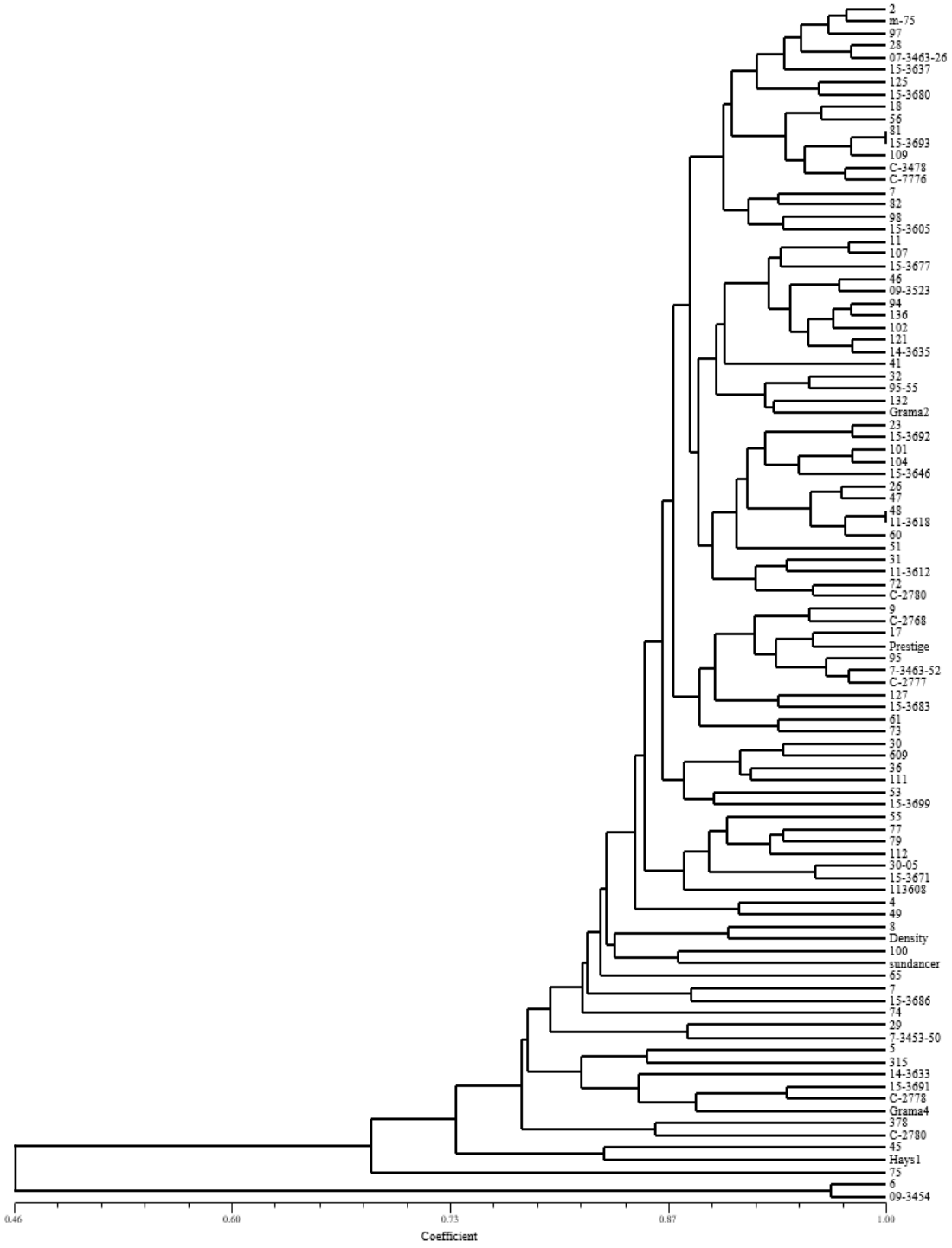

Figure 2.1. Unweighted pair-group arithmetic mean cluster analysis (UPGMA) dendrogram of newly collected buffalograss genotypes, elite breeding material and named buffalograss cultivars based on 24 simple sequence repeat markers. Coefficient represents the similarity coefficient between entries along the x-axis.

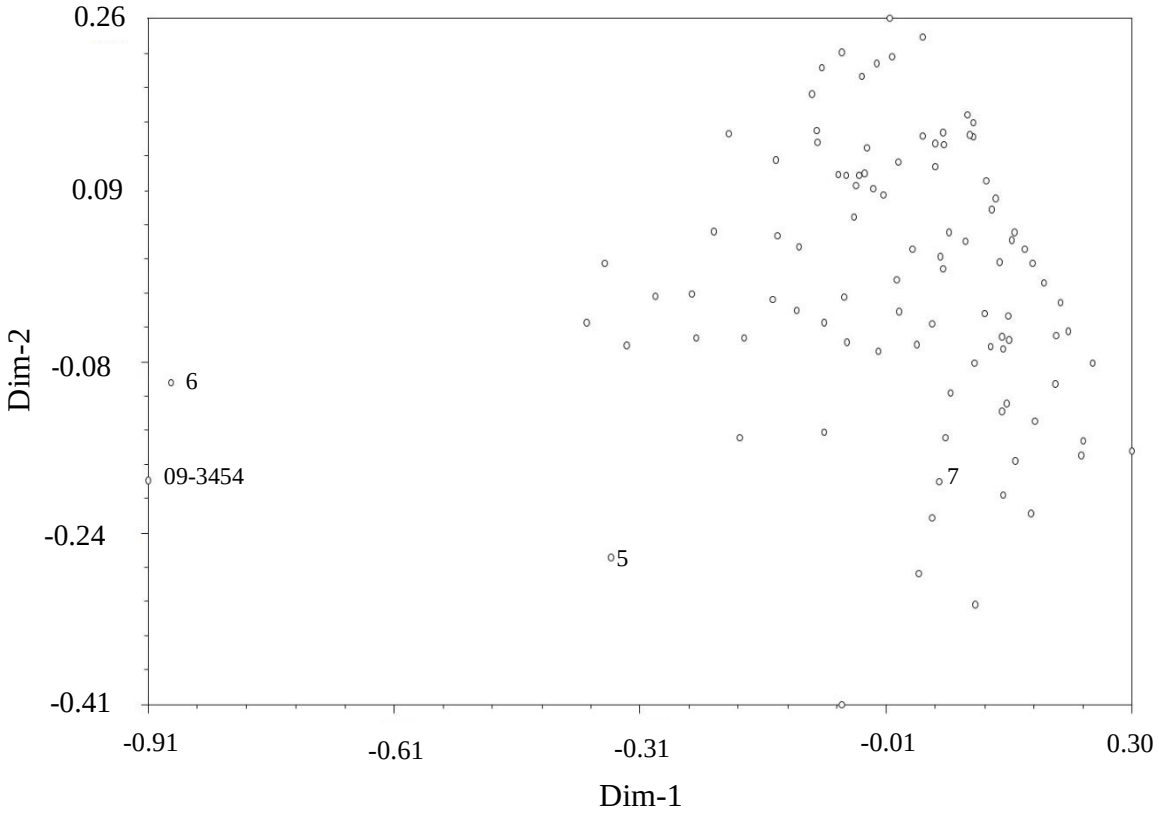

Figure 2.2. Principal component analysis (PCA) of 98 buffalograss genotypes. Large degrees of genetic similarity are plotted along the x-axis (Dim-1), and smaller degrees of genetic similarity are plotted along the y-axis (Dim-2). PCA was produced using the eigen function tools in NTSYSpcC.2.21. Entries referenced in the text are labelled.

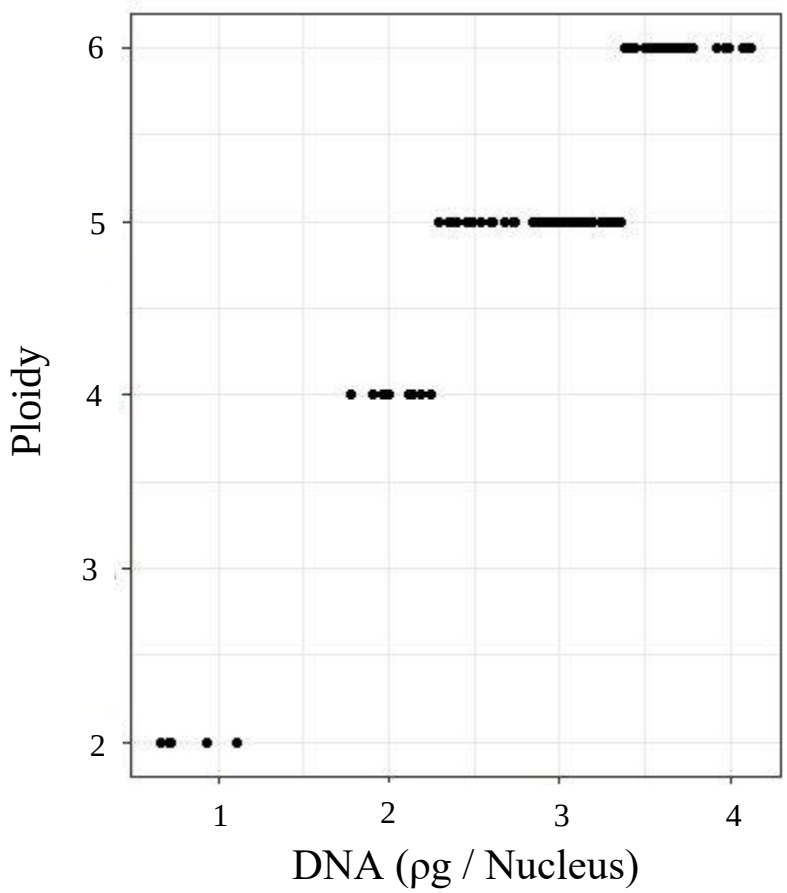

Figure 2.3. Ploidy determination of 58 buffalograss genotypes produced by DNA quantification using flow cytometry. DNA quantified by picograms per nucleus is represented along the horizontal axis and ploidy is represented along the vertical axis.

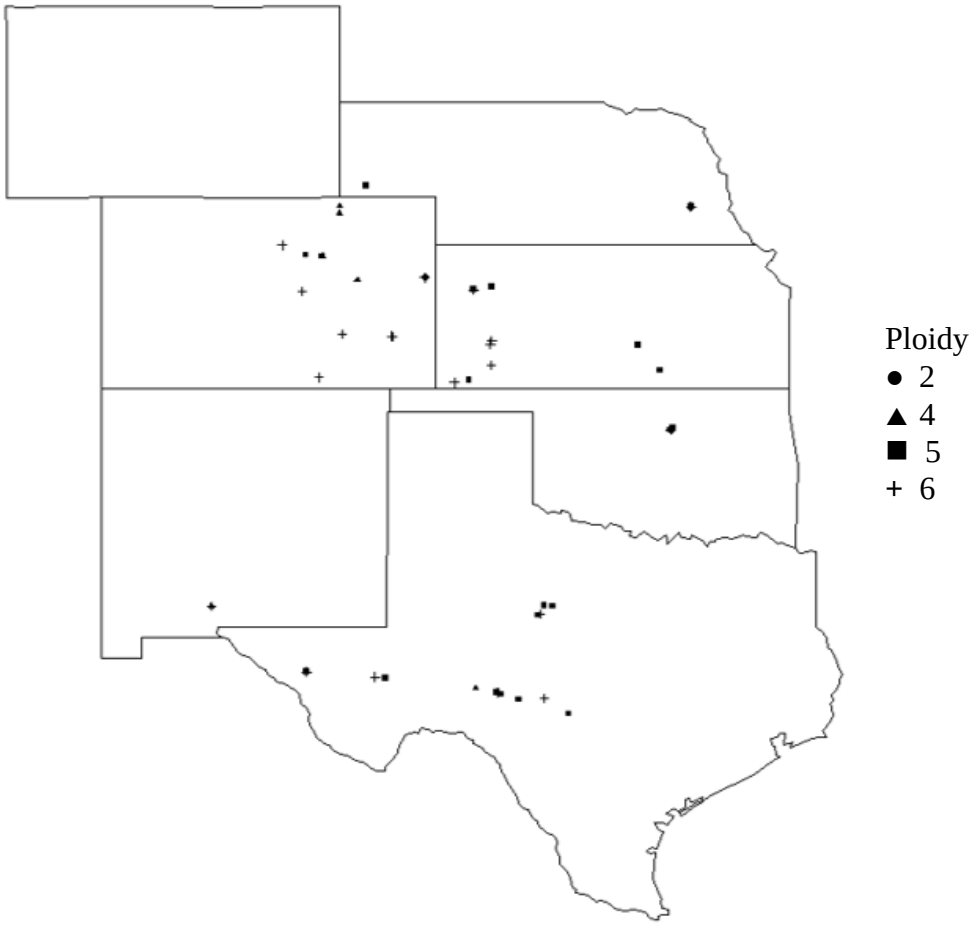

Figure 2.4. Ploidy of buffalograss samples collected and plotted by GPS location of collection site. Diploid buffalograsses are represented by filled circles, tetraploid buffalograsses are represented by filled triangles, pentaploid buffalograsses are represented by filled squares, and hexaploid samples are represented by crosses.

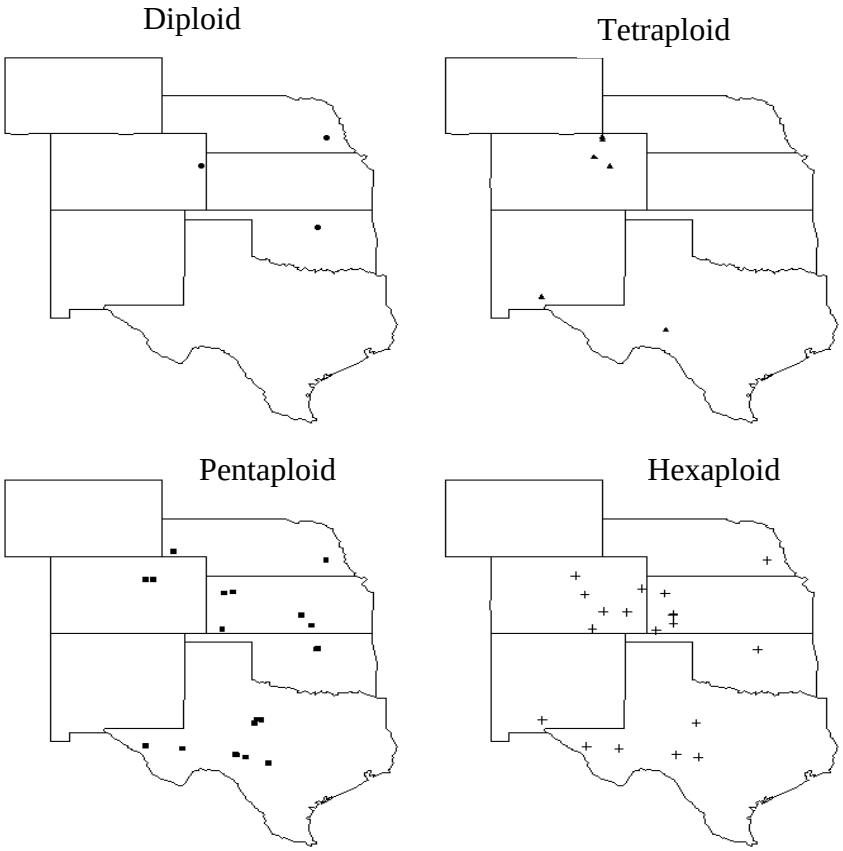

Figure 2.5. Buffalograss ploidy of 58 collected buffalograss genotypes. Distribution of the genotypes was split by ploidy of sample through NE, KS, TX, NM, and CO.

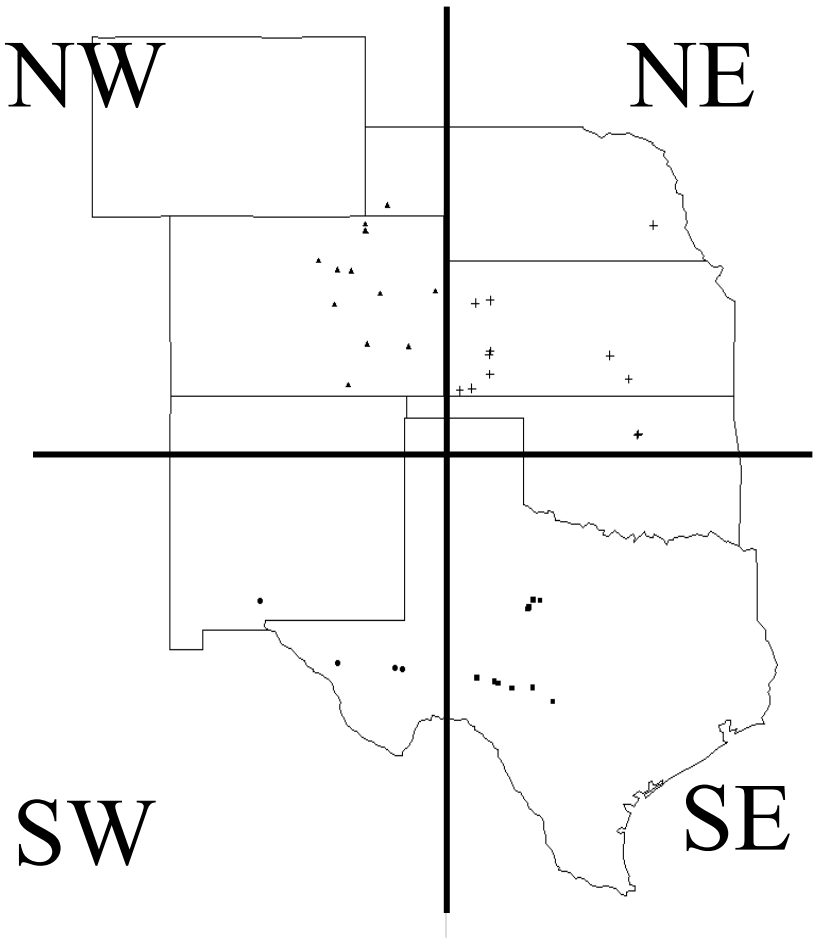

Figure 2.6. Mapping of a collection of 58 buffalograss genotypes for downstream analysis. The map was divided at latitude 35.7°N (horizontal axis) and longitude 102°W (vertical axis).

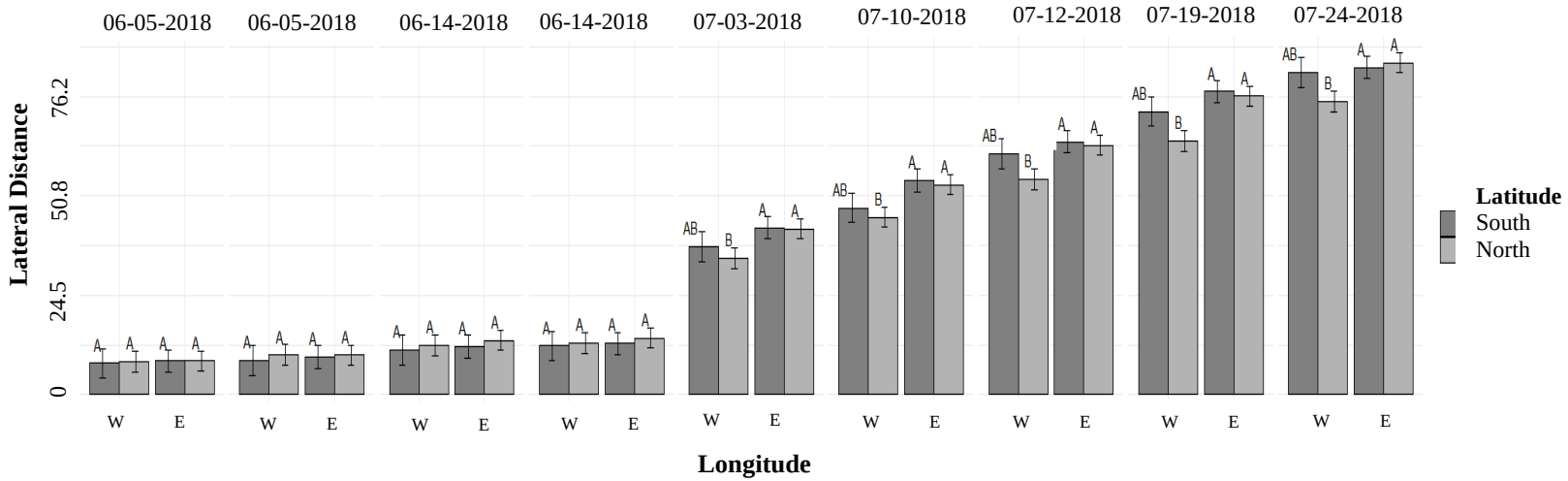

Figure 2.7. Fisher's LSD was performed on the 58 buffalograss genotypes testing lateral spread in inches based on longitudinal location over time. Lateral spread was measured on nine evaluation dates. Latitude is represented by two groupings as north or south of 35.7°N. Longitude is represented by two groupings as east or west of 102°W. Buffalograsses were evaluated in Lincoln, Nebraska.

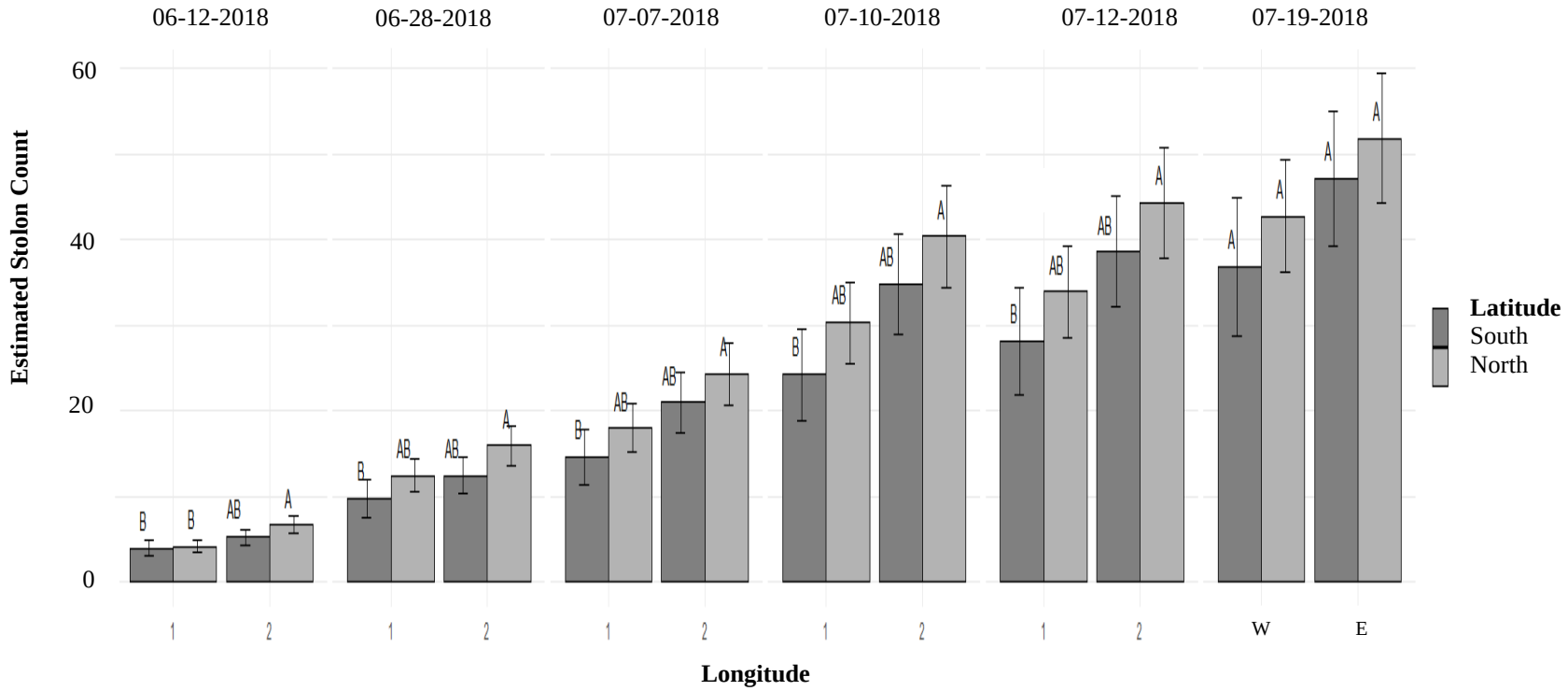

Figure 2.8. Count of fifty-eight individual buffalograss stolons with more than three nodes on six evaluation days. The data is represented by geographic location north or south of 35°N and longitude east or west of 102°W. Buffalograsses were evaluated in Lincoln, Nebraska.

Table 2.2. Analysis of variance for lateral spread over time was performed on 58 buffalograss genotypes. Buffalograss genotypes were evaluated in Lincoln, Nebraska.

Effect	Num DF	Den DF	F Value	Pr < F
Genotype	57	114	1.72	0.0072
Date	8	926	550.00	<0.0001
Genotype*Date	456	926	1.11	0.0893

Table 2.3. Analysis of variance for lateral spread in centimeters, was performed on 58 buffalograss entries. Ratings were analyzed based on geographic location and date after planting. Latitude represents two grouping of samples as north or south of 35.7°N. Longitude represents two grouping of samples as east or west of 102°W. Evaluations of buffalograss entries were made in Lincoln, Nebraska

Effect	Num DF	Den DF	F Value	Pr < F
Latitude	1	165	0.46	0.4982
Longitude	1	165	5.09	0.0253
Latitude * Longitude	1	165	0.55	0.4611
Date	8	1334	1082.16	<0.0001
Date* Latitude	8	1334	1.60	0.1207
Date* Longitude	8	1334	3.46	0.0006
Date* Latitude * Longitude	8	1334	0.92	0.4979

Table 2.4. Fishers LSD with Tukey's adjustment was performed on a collection of fifty-eight buffalograss accessions displaying the top and bottom performing buffalograss genotypes based on lateral distance in centimeters. Phenotypic evaluation was performed in Lincoln Nebraska.

Genotype	Estimate	Grouping	Genotype	Estimate	Grouping
81	47.333	A	72	32.66	ABCDE
46	40.666	BA	5	32.66	ABCDE
8	40.666	BA	45	31.66	ABCDE
11	40.00	ABC	56	31.66	ABCDE
94	39.33	ABC	82	31.33	ABCDE
60	39.33	ABCD	95-55	31.33	ABCDE
48	39.00	ABCD	79	31.00	ABCDE
4	38.33	ABCD	102	30.66	ABCDE
73	38.00	ABCD	378	30.66	ABCDE
47	38.00	ABCD	96	30.33	ABCDE
75	37.33	ABCD	315	29.66	BCEDE
125	36.33	ABCD	28	29.00	BCDE
109	36.00	ABCD	77	29.00	BCDE
7	36.00	ABCD	127	28.66	BCDE
74	36.00	ABCD	Sundancer	28.33	BCDE
Density	35.66	ABCD	609	28.00	BCDE
17	35.00	ABCDE	111	27.66	BCDE
136	34.66	ABCDE	30	27.66	BCDE
36	34.66	ABCDE	31	27.33	BCDE
55	34.33	ABCDE	23	27.00	BCDE
49	34.33	ABCDE	97	26.66	BCDE
32	34.00	ABCDE	95	25.00	BCDE
26	33.66	ABCDE	98	24.33	BCDE
61	33.66	ABCDE	51	23.00	CDEF
9	33.66	ABCDE	112	23.00	EDC
65	33.33	ABCDE	121	22.00	EDF
53	33.00	ABCDE	100	18.00	EF
Hays1	33.00	ABCDE	101	17.33	F
41	32.66	ABCDE			

Table 2.5. Analysis of variance for stolon count was performed on fifty-eight buffalograss accessions. Stolons with three or more nodes were counted. Buffalograss evaluations were made in Lincoln, Nebraska.

Effect	Df	Sum Sq	Mean Sq	F value	Pr>F
Genotype	57	38617	677.5	1.615	0.0153

Table 2.6. Analysis of variance for stolon width (μm) was performed on fifty-eight buffalograss accessions. Buffalograsses were evaluated in Lincoln, Nebraska using a caliper after the third node proximal to the center of the plot.

Effect	Num DF	Den DF	F Value	Pr < F
Genotype	57	114	1.58	0.0196

Table 2.7. Analysis of variance for stolon count was performed on fifty-eight buffalograss accessions. Stolons with more than three nodes were counted on an individual plant. investigating of stolon count based on evaluation date, latitudinal location, and longitudinal collection site location. Latitude represents two grouping of samples pooled be north or south of 35.7°N. Longitude represents two grouping of samples as east or west of 102°W. Buffalograsses were evaluated in Lincoln, Nebraska.

Effect	Num DF	Den DF	F Value	Pr < F
Latitude	1	165	1.58	0.2110
Longitude	1	165	4.82	0.0295
Latitude * Longitude	1	165	0.00	0.9745
Date	5	835	773.05	<.0001
Date* Latitude	5	835	0.79	0.5572

Table 2.8. Fishers LSD with Tukey's adjustment for stolon count of fifty-eight buffalograss accessions. Stolon counts were collected on 7/10/2018 in Lincoln Nebraska.

Sample	Estimate	Grouping	Sample	Estimate	Grouping
46	4.56	A	65	3.63	AB
378	4.17	AB	36	3.62	AB
136	4.15	AB	73	3.62	AB
60	4.14	AB	94	3.59	AB
61	4.08	AB	9	3.58	AB
4	4.04	AB	127	3.57	AB
81	4.03	AB	72	3.54	AB
8	4.01	AB	28	3.52	AB
111	4.00	AB	79	3.51	AB
Density	4.00	AB	102	3.46	AB
75	4.00	AB	125	3.39	AB
82	3.94	AB	56	3.33	AB
49	3.92	AB	112	3.30	AB
11	3.92	AB	132	3.25	AB
77	3.88	AB	hays1	3.20	AB
32	3.84	AB	98	3.20	AB
41	3.84	AB	121	3.09	AB
97	3.83	AB	7	3.07	AB
47	3.83	AB	23	3.04	AB
48	3.80	AB	55	2.85	AB
96	3.74	AB	45	2.83	AB
315	3.74	AB	31	2.82	AB
17	3.73	AB	Sundancer	2.78	AB
95-55	3.71	AB	53	2.63	AB
109	3.60	AB	609	2.59	AB
95	3.68	AB	100	2.53	AB
26	3.67	AB	30	2.40	AB
74	3.66	AB	51	1.74	B
5	3.66	AB	101	1.65	B

Table 2.8. Analysis of Variance for stolon width was performed on fifty-eight buffalograss accessions testing for differences in stolon width (μm) based on geographic location of collection site. Latitude represents two grouping of samples north or south of 35.7°N. Latitude represents two grouping of samples east or west of 102°W. Buffalograsses were evaluated in Lincoln, Nebraska.

Effect	Num DF	Den DF	F Value	Pr < F
Latitude	1	165	0.04	0.8457
Longitude	1	165	0.01	0.9147
Latitude*Longitude	1	165	1.36	0.2452

Table 2.9. Fishers LSD displaying top and bottom significance group of fifty-eight buffalograss entries separating groups based on stolon width (μm). Buffalograsses were evaluated in Lincoln, Nebraska.

Genotype	Estimate	Grouping	Genotype	Estimate	Grouping
47	54.33	AB	112	38.00	ABC
hays1	51.00	AB	7	37.33	ABC
31	50.67	ABC	73	37.00	ABC
100	47.00	ABC	127	36.67	ABC
56	46.67	ABC	82	36.67	ABC
95	45.67	ABC	136	36.67	ABC
77	44.67	ABC	Sundancer	36.33	ABC
102	44.67	ABC	65	36.33	ABC
132	43.00	ABC	315	36.33	ABC
9	42.67	ABC	5	36.00	ABC
98	42.67	ABC	55	36.00	ABC
49	42.00	ABC	32	36.00	ABC
109	42.00	ABC	111	36.00	ABC
41	41.67	ABC	17	35.67	ABC
97	41.67	ABC	121	35.67	ABC
95-55	41.67	ABC	11	35.67	ABC
60	41.00	ABC	75	35.33	ABC
30	41.00	ABC	36	34.67	ABC
48	40.67	ABC	23	34.00	ABC
26	40.00	ABC	46	33.67	ABC
125	40.00	ABC	51	33.33	ABC
72	39.33	ABC	378	33.33	ABC
96	39.33	ABC	74	33.33	ABC
61	39.00	ABC	28	32.67	ABC
4	38.67	ABC	Density	29.00	ABC
8	38.67	ABC	53	28.33	ABC
79	38.00	ABC	609	25.67	BC
81	38.00	ABC	45	25.33	BC
94	38.00	ABC	101	23.33	C

Table 2.10. Analysis of variance for visual quality of fifty-eight buffalograss accessions. Buffalograss visual quality were rated on a one to nine scale with a rating of one indicating poor overall uniformity, poor lateral spread, sparse canopy, and being brown or tan. Plots scoring a nine had rich green color, high uniformity, good lateral spread, and a dense canopy. Evaluations of these entries took place in Lincoln, Nebraska.

Effect	Df	Sum Sq	Mean Sq	F value	Pr>F
Genotype	57	217.9	3.822	1.755	0.00551

Table 2.11. Analysis of variance for visual quality based on geographic collection site of fifty-eight buffalograss accessions. Latitude represents two groupings of samples by being north or south of 35.7°N. Longitude represents two grouping of samples by being east or west of 102°W. Visual quality was rated on a one to nine scale, with a rating of one indicating a brown or tan color, poor uniformity, poor lateral spread, and sparse canopy density and a nine rating having a rich green color, good uniformity, wide lateral spread, and dense canopy. Buffalograss evaluations took place in Lincoln, Nebraska.

Effect	Df	Sum Sq	Mean Sq	F value	Pr > F
Latitude	1	13.5	13.514	5.06	0.0258
Longitude	1	0	0.004	0.001	0.9704
Latitude*Longitude	1	2.9	2.947	1.103	0.295

Table 2.12. Fishers LSD of visual quality for fifty-eight buffalograsses based on being collected north or south of 35.7°N. Visual quality was rated on a one to nine scale with a rating of one indicating a brown or tan color, poor uniformity, poor lateral spread, and sparse canopy density and a nine rating indicating a rich green color, good uniformity, wide lateral spread, and dense canopy. Buffalograss evaluations took place in Lincoln, Nebraska.

Latitude	Visual Quality	Groups
North	7.769231	a
South	7.175439	b

Table 2.13. Analysis of variance testing fall color of fifty-eight buffalograss accessions. Fall color was rated on a 1-9 scale with a ratings of one indicating a brown or light tan color and a rating of nine indicating the plot was completely green. Buffalograsses were evaluated in the fall of 2018 in Lincoln, Nebraska.

Effect	Num DF	Den DF	F Value	Pr < F
Genotype	56	112	1.5	0.0353
Date	2	228	815.33	<.0001
Genotype*Date	112	228	1.55	0.0027

Table 2.14. Fisher's LSD test for fall color of fifty-eight buffalograss accessions at three time points. Fall color was rated on a 1-9 scale with ratings of one indicating brown or light tan color and nine indicating the plot was completely green. Buffalograsses were evaluated in the fall of 2018 in Lincoln, Nebraska.

Date	Estimate	Groups
10/26/2018	3.666667	a
11/02/2018	2.310345	b
11/16/2018	0.982759	c

Table 2.15. Analysis of Variance testing fall color of buffalograss by locations north or south of 35.7°N, east or west of 102°W, and by date. Fall color was rated on a one to nine scale with ratings of one indicating brown or light tan color and nine indicating the plot was completely green. Buffalograsses were evaluated in Lincoln, Nebraska.

Effect	Num DF	Den DF	F Value	Pr < F
Latitude	1	165	0.22	0.6415
Longitude	1	165	4.41	0.0372
Latitude * Longitude	1	165	0.02	0.8984
Date	2	334	530.95	<.0001
Date* Latitude	2	334	1.68	0.1874
Date* Longitude	2	334	2.46	0.0873
Date* Latitude * Longitude	2	334	0.73	0.4815

Table 2.16. Fishers LSD test for fall color in newly collected buffalograss genotypes between samples west or east of 102°W. Fall color was rated on a one to nine scale with ratings of one indicating a brown or light tan color and nine indicating the plots were completely green. Buffalograsses were evaluated in Lincoln, Nebraska on 11/02/2018.

Longitude	Estimate	Groups
East	3.109524	a
West	2.804348	b

Table 2.17. Fishers LSD grouping of fifty-eight buffalograss genotypes evaluated for fall color. Fall color was rated on a one to nine scale with ratings of one indicating brown or light tan color and nine indicating the plots were completely green. Buffalograsses were evaluated in Lincoln, Nebraska on 11/02/2018.

Genotype	Estimate	Groups	Genotype	Estimate	Groups
112	4	a	102	2	ab
7	3.666667	ab	109	2	ab
30	3.333333	ab	125	2	ab
49	3.333333	ab	23	2	ab
55	3.333333	ab	36	2	ab
95	3.333333	ab	378	2	ab
79	3	ab	41	2	ab
Density	3	ab	46	2	ab
121	2.666667	ab	47	2	ab
17	2.666667	ab	53	2	ab
32	2.666667	ab	60	2	ab
48	2.666667	ab	61	2	ab
5	2.666667	ab	73	2	ab
56	2.666667	ab	74	2	ab
609	2.666667	ab	77	2	ab
82	2.666667	ab	8	2	ab
9	2.666667	ab	96	2	ab
94	2.666667	ab	97	2	ab
11	2.333333	ab	98	2	ab
111	2.333333	ab	hays1	2	ab
132	2.333333	ab	100	1.666667	ab
136	2.333333	ab	101	1.666667	ab
26	2.333333	ab	127	1.666667	ab
28	2.333333	ab	4	1.666667	ab
31	2.333333	ab	51	1.666667	ab
315	2.333333	ab	72	1.666667	ab
45	2.333333	ab	75	1.666667	ab
95-55	2.333333	ab	81	1.666667	ab
Sundancer	2.333333	ab	65	1.333333	b

Table 2.18. Analysis of Variance testing spring color of fifty-eight buffalograss accessions on 4/19/2019. Spring color was rated on a one to nine scale with ratings of one indicating brown or light tan color and nine indicating the plots were completely green. Buffalograss evaluations were made in Lincoln, Nebraska.

Effect	Num DF	Den DF	F Value	Pr < F
Genotype	57	114	3.47	<0.0001

Table 2.19 Analysis of Variance testing spring color of fifty-eight buffalograss accessions 4/25/2019. Spring color was rated on a one to nine scale with ratings of one indicating brown or light tan color and nine indicating the plots were completely green. Buffalograss evaluations were made in Lincoln, Nebraska.

Effect	Df	Sum Sq	Mean Sq	F value	Pr >F
Genotype	57	264.7	4.643	2.184	0.000197

Table 2.20. Fishers LSD of spring green up for fifty-eight buffalograss genotypes on 4/25/2019. Buffalograsses were evaluated in Lincoln, Nebraska.

Genotype	Estimate	Group	Genotype	Estimate	Group
65	4.666667	a	101	3	abc
94	4.666667	a	109	3	abc
55	4.333333	ab	11	3	abc
100	4	abc	112	3	abc
48	4	abc	125	3	abc
75	4	abc	136	3	abc
102	3.666667	abc	23	3	abc
111	3.666667	abc	28	3	abc
121	3.666667	abc	378	3	abc
132	3.666667	abc	4	3	abc
315	3.666667	abc	41	3	abc
81	3.666667	abc	46	3	abc
82	3.666667	abc	49	3	abc
9	3.666667	abc	56	3	abc
95	3.666667	abc	60	3	abc
95-55	3.666667	abc	61	3	abc
hays1	3.666667	abc	7	3	abc
127	3.333333	abc	73	3	abc
17	3.333333	abc	8	3	abc
31	3.333333	abc	98	3	abc
32	3.333333	abc	26	2.666667	abc
36	3.333333	abc	5	2.666667	abc
47	3.333333	abc	96	2.666667	abc
72	3.333333	abc	30	2.333333	abc
74	3.333333	abc	53	2.333333	abc
77	3.333333	abc	Density	2.333333	abc
79	3.333333	abc	45	2	bc
97	3.333333	abc	609	2	bc
Sundancer	3.333333	abc	51	1.666667	c

Table 2.21. Analysis of Variance for spring color in Lincoln, Nebraska based on geographic information of fifty-eight buffalograss collection sites. Spring color was rated on a one to nine scale with ratings of one indicating brown or light tan color and nine indicating the plots were completely green. Latitude represents two groupings of samples by north or south of 35.7°N. Longitude represents two groupings of samples by east or west of 102°W.

Effect	Num DF	Den DF	F Value	Pr < F
Latitude	1	168	12.27	0.0006
Longitude	1	168	0	0.9587
Latitude * Longitude	1	168	0.3	0.5853
Date	1	170	473.53	<.0001
Date* Latitude	1	170	0.09	0.7601
Date* Longitude	1	170	2.34	0.1277
Date* Latitude *Longitude	1	170	0.42	0.5199

Table 2.22. Fishers LSD testing spring color on 4/25/2019 of fifty-eight buffalograss accessions based on latitude of collection site in Lincoln, Nebraska. Spring color was rated on a one to nine scale with ratings of one indicating brown or light tan color and nine indicating the plots were completely green. Latitude represents two groupings of samples by north or south of 35.7°N.

Latitude	Estimate	groups
North	2.431624	A
South	2.140351	B