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Exploration of Genetic Diversity and Associations Between Genotype and Phenotype in *Cynodon* spp.

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ABSTRACT OF THE DISSERTATION

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Rising global temperatures and the decreasing amount of water available for irrigating crops has become a major concern, especially in the turfgrass industry. These trends highlight the need adopt turfgrasses that are better adapted to both current and future climates. Bermudagrasses (*Cynodon* (L.) Rich) are warm-season grasses that are well-adapted to warm and dry climates, making them an ideal turfgrass for regions where water may be scarce. The genus also boasts a large range of genetic diversity, which can be leveraged to improve consumer- and agronomic-related traits, such as winter color retention. However, genomic resources are lacking for bermudagrass and distinguishing between species can be difficult given their phenotypic plasticity and morphological variation. This makes it difficult to effectively utilize the genetic diversity available in germplasm collections for breeding. In this dissertation, I first examined the systematics of the *Cynodon* genus and proposed the reclassification of several accessions collected from publicly available germplasm repositories. Exploratory work was done to identify and develop genetic markers that may aid breeders and taxonomists in identifying some of the bermudagrass species. Next, I evaluated the performance of inter- and intraspecific

bermudagrass hybrid accessions under prolonged and repeated drought conditions using a novel method. Results from this study suggest the presence of different types of stress memory among the accessions, suggesting that this method may be useful in both selecting for improved drought tolerance as well as enhancing the drought tolerance of established stands. Lastly, I conducted a genome-wide association study (GWAS) to identify markers associated with winter color retention in a population of bermudagrasses. Despite several limiting factors in the study, such as admixture and differences in ploidy throughout the population, results from the GWAS identified two significant DNA polymorphisms, with one aligning to a gene known to be involved in cold stress response in maize. This dissertation emphasizes the need to develop more genomic resources for bermudagrass in order to select for accessions with improved consumer- and agronomic-related traits.

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Introduction

Bermudagrass is the most common name used in reference to several taxa of grasses from the genus *Cynodon* (L.) Rich. Bermudagrasses are perennial, warm-season grasses widely used as turf as they form a uniform ground cover while tolerating low mowing and regular traffic. They are typically used for homeowner and commercial lawns, parks, athletic fields, and golf courses. Considered a noxious weed in the past (Fernandez 2003), continued hybridization and recurrent selection for turf characteristics has pushed it to become one of the most widely used turfgrass in the United States. It is naturally adapted to warmer and drier climates, increasing its value as a sustainable turf in the face of global climate change. Although many *Cynodon* species originated from, and are adapted to, warm tropical and subtropical regions such as Africa, accessions have been collected from regions as far north as 53°N latitude and at elevations of 3000 m (Hanna, Raymer, and Schwartz 2013; Taliaferro 2003), highlighting its wide geographic range of distribution and adaptations, and potentially large amount of genetic diversity within the genus.

Cynodon is a member of the family *Poaceae*, subfamily *Chloridoideae*, tribe *Cynodonteae*, and subtribe *Chloridinae* (Jack Rodney Harlan et al. 1970). The taxonomy of the *Cynodon* genus has undergone multiple revisions in the twentieth century to devise a satisfactory method of classification (Hurcombe 1948; de Wet and Harlan 1970). Difficulties in classifying the *Cynodon* taxa can be attributed to the large phenotypic variation within the genus. The variability,

both within and between species, often includes intermediate ecotypes with morphological similarities that pose challenges for species identification. Across latitudinal and longitudinal gradients, Zhang et al. (2018) and Wang et al. (2020) observed significant morphological variation among *C. dactylon* accessions collected in China. Wang et al. (2020) observed a cline of leaf and internode lengths from east to west, while Zhang et al. (2018) found traits, such as leaf length, internode length, turf height, and reproductive branch height, to be highly variable but overall larger at lower and higher latitudes, suggesting local adaptations to climate and environment.

Despite extensive studies, leading to a major revision of the *Cynodon* genus in 1970, the total number of bermudagrass species remains inconsistent across a variety of sources. Most literature, however, lists the following eight species: *C. aethiopicus* Clayton et Harlan, *C. arcuatus* J. S. Presl ex C. B. Presl, *C. barberi* Rang. et Tad., *C. dactylon* (L.) Pers., *C. incompletus* Nees, *C. nlemfuensis* Vanderyst, *C. plectostachyus* (K. Schum.) Pilg., and *C. transvaalensis* Burtt-Davy. *C.* x *magennisii* Hurcombe, often listed as a separate species, is a naturally occurring hybrid between *C. dactylon* and *C. transvaalensis,* a sterile triploid that can be used as turf via clonal propagation (Huffine 1957). Harlan et al. (1970), originally classified *C.* x *magennisii* as a separate taxon (Jack Rodney Harlan et al. 1970), but omitted it from the subsequent revision of the *Cynodon* genus around the same time (Harlan 1970; de Wet and Harlan 1970), presumably based on its narrow endemic region and

sterile nature. The Royal Botanic Gardens, Kew (1999) originally shared the same sentiment and listed eight species, also omitting *C.* x *magennisii*; however, they now recognize 14 *Cynodon* species, which includes *C.* x *magennisii* plus additional five species native to Australia or Java and New Guinea: *C. ambiguus* (Ohwi) P. M. Peterson, *C. convergens* F. Muell., *C. prostratus* (C. A. Gardner & C. E. Hubb.) P. M. Peterson, *C. simonii* P. M. Peterson, and *C. tenellus* R. Br. (https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:331181-2#children). The United States Department of Agriculture Natural Resources Conservation Service (USDA-NRCS; https://plants.usda.gov/home/plantProfile?symbol=CYNOD) lists ten species, which includes *C.* x *magennisii,* as well as an additional species not recognized by the Royal Botanical Gardens, Kew, *C. bradleyi* Stent. Recent literature varies in the proposed number of species (eight or nine), depending on the sources and their inclusion/exclusion of *C.* x *magennisii*.

The *Cynodon* taxa show distinct morphologies, ploidy levels, and sexual compatibility, and current classifications were assigned in de Wet and Harlan's (1970) biosystematic analysis that led to the aforementioned revision of the *Cynodon* genus taxonomy (de Wet and Harlan 1970). That classification was based on morphology, geographic distribution, ecological behavior, cytogenetic data, and sexual compatibility, and remains the most extensive study to date regarding the systematics of the genus. Even so, Harlan and de Wet note in multiple papers that "the genus *Cynodon* is difficult to treat taxonomically and

that no entirely satisfactory classification is possible" (Jack R. Harlan et al. 1970). This sentiment is echoed elsewhere throughout the literature and highlights the need for careful consideration in assigning *Cynodon* species (Chiavegatto et al. 2016).

Bermudagrass species have the base chromosome number of x = 9, their ploidy levels range from diploid (2n = 2x = 18) to hexaploid (2n = 6x = 54), though pentaploids and hexaploids are sometimes observed (Taliaferro 2003). A brief description of each species is as follows. *C. aethiopicus* Clayton and Harlan has inflorescences with 1–3 whorls of stiff, erect racemes and woody stolons, is distributed in East Africa, and occurs as a diploid or tetraploid (Clayton and Harlan 1970). *C. arcuatus* J. S. Presl ex C. B. Presl. is morphologically distinct in its large ovate-lanceolate leaf shape, lack of rhizomes, flexuous racemes (3-6) in one whorl, and is found in Malagasy, India, South East Asia, and northern Australia; it is tetraploid (Bosser 1966). *C. barberi* Rang. et Tad. is characterized by glumes that are approximately the length of the spikelets, short racemes (3-6) in one whorl, small ovate-lanceolate leaves, and a lack of rhizomes. It is endemic to South India as a diploid species (Jack R. Harlan et al. 1970; de Wet and Harlan 1970). *C. dactylon* (L.) Pers. is a highly variable species, ranging from fine-textured leaves that are suitable for turf, to large, coarse leaves with high biomass production suitable for forage (Harlan and de Wet 1969). They are mostly rhizomatous, have 3-10 racemes in one whorl, and are distributed in Afghanistan, South Africa, and Madagascar (de Wet and Harlan 1970). They can

be diploid or tetraploid. *C. incompletus* Nees has short, hairy leaves and lacks rhizomes, and is endemic to South Africa as a diploid or tetraploid (de Wet and Harlan 1970). *C. nlemfuensis* Vanderyst has broad leaves and woody stolons, lacks rhizomes, and occurs in East Africa as a diploid or tetraploid (Clayton and Harlan 1970; Jack R. Harlan et al. 1970). *C. plectostachyus* (K. Schum.) Pilger has large, broad, hairy leaves, no rhizomes, and is endemic to East Africa as a diploid species (de Wet and Harlan 1970). Finally, *C. transvaalensis* Burtt-Davy is characterized by small, fine-textured leaves, small inflorescences, spikelets loosely arranged on three racemes per whorl, and red pigmentation of stems in the cold; it is endemic to South Africa as a diploid species (de Wet and Harlan 1970). Both *C. dactylon* and *C. transvaalensis* can be considered as the major species of the genus, as they are the primary species used in turfgrass breeding today. The other *Cynodon* species are less common in bermudagrass breeding programs and have played a minor role in cultivar development.

Generally, tests of ploidy levels are quite simple. Not so in Cynodon. Here, both classical and modern cytogenetic techniques, such as chromosome counts on root-tip squash preparations, and flow cytometry, have been used to determine the ploidy levels of individual accessions. Flow cytometry has been reported as a reliable method for estimating ploidy (Taliaferro 2003) and is commonly cited in recent literature. Mean 2C nuclear DNA contents ranges have been reported for *C. transvaalensis* and *C. dactylon* as 1.10 to 1.14 pg and 1.96 to 2.30 pg, respectively (Taliaferro et al. 1997; Wu et al. 2006). With such large

differences in DNA content, it would be next to impossible to distinguish triploid interspecific hybrids from natural tetraploids. Here, Grossman et al. (2021) pointed out the importance of chromosome counts as the range of DNA contents can be wide (Grossman et al. 2021). A similar stance was taken by Karaca et al. (2000) and is supported by Zhang et al. (2019), who reported substantial variation in genome sizes among tetraploid *C. dactylon* accessions (Karaca et al. 2000; Zhang, Wang, Guo, Guan, Guo, et al. 2019). Few cytogenetic studies have included the minor *Cynodon* taxa as per Harlan's revision of the *Cynodon* taxonomy. To date, only Chiavegatto et al. (2016) lists both the chromosome counts and flow cytometry data for accessions belonging to the minor taxa. While some might consider chromosome counting tedious, it is an important piece of the taxonomic puzzle when assigning an accession's species identity.

Of the minor taxa, several are sexually compatible amongst one another while others are genetically isolated. Diploid *C. aethiopicus* are capable of producing hybrids with *C. nlemfuensis,* while tetraploid *C. aethiopicus* can hybridize with *C. dactylon*; diploid *C. nlemfuensis* is compatible with *C. transvaalensis*; and *C. incompletus* is compatible with *C. dactylon* (de Wet and Harlan 1970). *C. acruatus*, *C. barberi*, and *C. plectostachyus* vary in their isolation from the other taxa and have not been reported to be compatible with either of the two major taxa, *C. dactylon* and *C. transvaalensis*. Given that three of the six minor taxa are compatible with at least one of the two major taxa, serious consideration should be given to including them into turf breeding

programs. Two of the minor taxa, *C. arcuatus* and *C. plectostachyus*, have found use as forage with minimal use as a turf species. Hanna et al. (2013) and Taliaferro (2003) point out that there may be potential in including minor *Cynodon* taxa in turf breeding programs as donors of resistance or tolerance genes, yet few studies have included them. Hanna et al. (2013) goes on to claim that “there is probably enough variation in current [bermudagrass] collections to last for a lifetime of breeding”, but they do now appear to include the minor taxa in this presumption (Hanna et al. 2013).

Phylogenetics, paired with genetic markers, have been used in more recent attempts to distinguish and group individuals of a particular species across the plant kingdom. Most of these studies in *Cynodon*, however, have focused on only the two major taxa, *C. dactylon* and/or *C. transvaalensis*, presumably for their importance to the turf industry. For *C. dactylon* in particular, many studies highlight the large amount of genetic diversity within the species. Phylogenetic and genetic diversity analyses have shown that *C. dactylon* accessions may cluster together based on their geographic origin (Huang et al. 2010; Ling et al. 2015; Singh et al. 2023; Wu et al. 2004). They have also shown that large amounts of diversity between *C. dactylon* accessions may still exist within any one region across latitudinal gradients (Etemadi et al. 2006; Huang et al. 2014; Kang et al. 2008; Pudzianowska and Baird 2021; Singh et al. 2023; Zhang, Wang, Guo, Guan, Liu, et al. 2019), further demonstrating the massive diversity within this cosmopolitan species. Interestingly, in the few genetic diversity studies

that included the minor taxa, discrepancies have been found for several accessions between the accession's phylogenetic clustering and their species assignment by collectors (Assefa et al. 1999; Jewell et al. 2012; Pudzianowska and Baird 2021). In contrast with the claims of genetic isolation made by Harlan et al. (1970), both Jewell et al. (2012) and Pudzianowska and Baird (2021) found the accessions of *C. barberi* and *C. radiatus* to group closely with a set of *C. dactylon* accessions. One might argue that these studies are limited, however, in their representation of each of the minor taxa when compared to the major taxa. This is not entirely surprising, as public germplasm repositories, such as the USDA-ARS NPGS, include very limited numbers of accessions from the minor *Cynodon* taxa, being heavily skewed towards the two major taxa. The general conclusions from this range of taxonomic/phylogenetic studies are: 1) morphological data alone are insufficient for the classification of *Cynodon* accession and must be supplemented with genetic, geographic, and cytogenetic data; and 2) there is a need for higher throughput methods (e.g. genetic markers) for species identification of existing germplasm collections.

Given the confusion and challenges surrounding *Cynodon* classifications, it is important that new tools (e.g. species-specific markers) be developed to aid in taxonomic studies and identifications. In the past two decades, genetic markers have been used to identify individual bermudagrass accessions. Several studies used simple sequence repeats (SSRs) (Godwin, Fang, and Wu 2020; Harris-Shultz, Schwartz, and Brady 2011; Wang et al. 2010; Yang et al. 2018).

SSRs are found throughout the genome as repeat motifs of nucleotides that vary in length, and the differences in the number of repeats can be detected through PCR-based methods and utilized as genetic markers. SSR markers are highly polymorphic, codominant, and have high reliability (Vieira et al. 2016). These “cultivar-specific” markers provide breeders with a tool to distinguish between cultivars without the need for relying on morphological traits. Another genetic marker commonly used are single nucleotide polymorphisms (SNPs), which are genome variations at a single base position at the DNA level, and are perhaps the most widely used type of genetic marker today. Similar to SSRs, SNP markers are also polymorphic, codominant, and have high reliability, but have not been used in bermudagrass for cultivar or accession differentiation. They are often used in tandem with PCR-based methods to develop primers for cultivar or species identification and have been applied in a variety of crops, such as juniper (García, Guichoux, and Hampe 2018), melon (Zhang et al. 2023), *Capsicum* (Jung et al. 2010), *Cucurbita* (Yoo et al. 2023), rice (Cheon et al. 2018; Ndjiondjop et al. 2018), and ryegrass (Pembleton et al. 2016).

Given the current status of taxonomy of *Cynodon*, and apparent confusion in species identification in the USDA collection (Pudzianowska and Baird 2021) one of the goals of the research presented here was to attempt clarification of these issues. To do so, chromosome counts, DNA content analysis via flow cytometry, and extensive genotyping were undertaken. As in previous studies, large differences in the DNA contents were noted; chromosome counts

demonstrated the presence of triploids; presumably spontaneous hybrids either collected in the wild and misclassified when placed in collections, or occurred in collections themselves where close proximity of small plots of various origins provide ample opportunity for cross hybridization. Seed produced in this manner would likely drop to the ground and germinate, where hybrids with sufficient vigor may over time replace the original parent. When all these data were combined, and triploids removed, a much clearer picture of species groupings within *Cynodon* was obtained. To advance this line of work even further, an exploratory study was done to test if species specific DNA markers could be identified and indeed first such markers were found.

Of the eight (or nine) taxa within the *Cynodon* genus, two are of particular importance (*C. dactylon* and *C. transvaalensis*) and are the predominant species used in breeding for turf. Most turf-type bermudagrasses of economic value are either derived from interspecific hybridizations between tetraploid *C. dactylon* and diploid *C. transvaalensis*, or from *C. dactylon* clones. Hybrids produced from crosses between tetraploid *C. dactylon* and *C. transvaalensis* are sterile triploids and generally demonstrate better quality and enhanced performance traits ideal for turf. Although there have been cultivars produced from hybridizations between a minor and major *Cynodon* taxon, these cultivars are no longer in use, replaced by improved hybrids derived from *C. dactylon* and *C. transvaalensis* crosses. Bermudagrass can be vegetatively propagated, instantly fixing the

characteristics of successful hybrids regardless of the level of heterozygosity they may carry.

Breeding programs of bermudagrass in the United States have historically focused on enhancing turf quality traits and only recently started to target abiotic and biotic resistance/tolerance. Turfgrasses are functional, sometimes ornamental, crops that rely heavily on their visual appeal and aesthetic. Thus, competitive or improved turf quality traits are necessary for successful adoption by consumers. Turf quality is an assessment applicable to all turfgrass species and is evaluated on a visual basis. Although quality ratings are relative within a species, it is a subjective assignment based on a combination of leaf color, density, uniformity, and texture (a visual assessment of leaf width), as well as response to abiotic/biotic stresses (Morris and Shearman 1998). Abiotic factors, such as drought and cold tolerance, have also become major objectives for improvement in bermudagrass.

Bermudagrass is naturally adapted to high temperatures and drought conditions, but there still exists a large amount of variation in reaction to both the temperatures and water availability that can be leveraged by breeding programs. The first observable drought stress symptoms are leaf wilting and firing. Occasional blue or purple discoloration may also occur and is thought to be due to the production of waxes or antioxidants, such as anthocyanins. Differences in drought tolerance between accessions have been studied (Katuwal et al. 2022; Shi et al. 2012; Zhou, Lambrides, and Fukai 2013), and ultimately highlight the

large amount of variation for breeding described by Hanna et al. (Hanna et al. 2013). Bermudagrass is especially adept at drought avoidance, and is able to do so through root traits, such as increased rooting depth and density (Carrow 1995, 1996; Qian, Fry, and Upham 1997), as well as through leaf traits, such as stomatal closure (Hu, Wang, and Huang 2009). Genetic studies characterizing stress responses in bermudagrass remain limited, as genomic resources have only been recently made available. Kim et al. (2009) identified 189 drought-responsive candidate genes in *C. dactylon*, of which 120 were up-regulated and 69 were down-regulated in response to stress. The up-regulated genes were associated with proline synthesis and transcriptional activation of ABA-associated genes (Kim, Lemke, and Paterson 2009). Zhou et al. (2014) characterized the gene expression of two bermudagrass cultivars, cv. Tifway (*C. dactylon* x *C. transvaalensis*) and C299 (*C. dactylon*), in response to drought stress and identified 277 drought responsive genes. Many of these genes belonged to drought avoidance traits (e.g. cuticle wax formation) or drought tolerance traits (eg. oxidative stress defense) (Zhou et al. 2014).

A part of the research presented here was designed to improve bermudagrass drought tolerance through selection and to observe the types of reactions to drought stress among various accessions. A drydown study was conducted over three years where accessions in a replicated trial were subjected to prolonged and repeated drought conditions, separated by brief recovery periods. This led to an interesting observation that some bermudagrasses may

positively react to stress priming, where an initial period of stress may prime accessions and mitigate the effects of subsequent periods of stress. This observation may have direct practical value for irrigation practices, perhaps saving substantial amounts of water.

Low temperatures are one of the major abiotic stresses that bermudagrass faces, and provides two important targets for breeders: improved cold tolerance and/or mitigating winter dormancy. Bermudagrass, like most warm-season grasses, enters dormancy in response to lower temperatures. Its turf quality declines as temperatures drop below 15°C, and dormancy may occur below 10°C (Huang et al. 2019). Dormancy onset can be visually characterized by reduced growth and discoloration of the above-ground tissue, from dark green to a straw-brown color. Such discoloration is viewed as unfavorable, as preferences for year-round green color can limit the acceptance of bermudagrass by consumers in southern states of the United States. At temperatures below 0°C, freezing damage can be incurred, resulting in winterkill of partial or entire stands. Freezing tolerance has been shown to improve in plants slowly acclimated to lower temperatures (Thomashow 2001) and has been studied in other turfgrass species (Dionne et al. 2001; Espevig et al. 2011) as well as in bermudagrass (Li et al. 2023; Zhang, Ervin, et al. 2011; Zhang, Wang, et al. 2011). Metabolic responses to drought and cold stress appear similar, such as increased proline content, increased antioxidant enzyme activity, and increased metabolite production (Huang et al. 2019). Several genetic studies have been

conducted on gene expression in response to cold stress with and without cold-acclimation (Fan et al. 2014, 2015; Hu et al. 2017, 2018; Zhu et al. 2015), with one of the key outcomes being that cold-acclimation studies are the most effective method of screening for improved cold tolerance.

Conclusions

The systematics of the *Cynodon* genus and the wide phenotypic variability of the *C. dactylon* taxon makes proper classification of accessions difficult. These challenges may impact breeders and affect downstream decisions when attempting to produce hybrids with improved traits. Discrepancies between species nomenclature as assigned by collectors and those observed through (quantitative) genetic methods, such as phylogenetics, have the potential to reduce or inflate the amount of genetic diversity within a germplasm collection, either through redundancies or species incompatibility. Developing methods to differentiate between taxonomic groups, such as species-specific genetic markers, would greatly benefit both taxonomists and breeders, and would reduce confusion. Additional genetic resources from next generation sequencing or genetic association studies may also improve the understanding of researchers and breeders alike when trying to enhance quality or abiotic/biotic stress resistance traits. As global climates continue to shift towards warmer and drier trends, it is important to continually improve and adopt the usage of plant species that may be better adapted to future environments. Bermudagrass appears to fit

this bill perfectly. By reducing its dormancy period in response to cold stress, bermudagrass may be more widely used and accepted by consumers. This, however, may result in additional challenges to overcome, as a reduction in the dormancy period may not translate into enhanced cold tolerance.

References

Assefa, S., Taliaferro, C. M., Anderson, M. P., de los Reyes, B. G., & Edwards, R. M. (1999). Diversity among Cynodon accessions and taxa based on DNA amplification fingerprinting. Genome, 42(3), 465–474. https://doi.org/10.1139/g98-152

Bosser, J. (1966). Note sur les Graminees de Madagascar. IV. Cynodon A. Rich. A Madagas-car. Adansonia, 6, 247–250.

Carrow, R. N. (1995). Drought Resistance Aspects of Turfgrasses in the Southeast: Evapotranspiration and Crop Coefficients. Crop Science, 35(6), 1685–1690. https://doi.org/10.2135/cropsci1995.0011183X003500060029x

Carrow, R. N. (1996). Drought Resistance Aspects of Turfgrasses in the Southeast: Root-Shoot Responses. Crop Science, 36(3), cropsci1996.0011183X003600030028x. https://doi.org/10.2135/cropsci1996.0011183X003600030028x

Cheon, K.-S., Baek, J., Cho, Y., Jeong, Y.-M., Lee, Y.-Y., Oh, J., Won, Y. J., Kang, D.-Y., Oh, H., Kim, S. L., Choi, I., Yoon, I. S., Kim, K.-H., Han, J.-H., & Ji, H. (2018). Single Nucleotide Polymorphism (SNP) Discovery and Kompetitive Allele-Specific PCR (KASP) Marker Development with Korean Japonica Rice Varieties. Plant Breeding and Biotechnology, 6(4), 391–403. https://doi.org/10.9787/PBB.2018.6.4.391

Chiavegatto, R. B., Paula, C. M. P., Souza Sobrinho, F., Benites, F. R. G., & Techio, V. H. (2016). Karyotype asymmetry in Cynodon Rich. (Poaceae) accessions. Genetics and Molecular Research, 15(4). https://doi.org/10.4238/gmr15049152

Clayton, W. D., & Harlan, J. R. (1970). The Genus Cynodon L.C. Rich. In Tropical Africa. Kew Bulletin, 24(1), 185. https://doi.org/10.2307/4103262

Cynodon Rich. | Plants of the World Online | Kew Science. (n.d.). Plants of the World Online. Retrieved February 15, 2024, from http://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:331181-2

de Wet, J. M. J., & Harlan, J. R. (1970). Biosystematics of Cynodon L. C. Rich. (Gramineae). TAXON, 19(4), 565–569. https://doi.org/10.2307/1218950

Dionne, J., Castonguay, Y., Nadeau, P., & Desjardins, Y. (2001). Freezing Tolerance and Carbohydrate Changes during Cold Acclimation of Green-Type Annual Bluegrass (Poa annua L.) Ecotypes. Crop Science, 41(2), 443–451. https://doi.org/10.2135/cropsci2001.412443x

Espevig, T., DaCosta, M., Hoffman, L., Aamlid, T. S., Tronsmo, A. M., Clarke, B. B., & Huang, B. (2011). Freezing Tolerance and Carbohydrate Changes of Two Agrostis Species during Cold Acclimation. Crop Science, 51(3), 1188–1197. https://doi.org/10.2135/cropsci2010.07.0415

Etemadi, N., Sayed-Tabatabaei, B. E., Zamanni, Z., Razmjoo, K., Khalighi, A., & Lessani, H. (2006). Evaluation of diversity among Cynodon dactylon (L.) Pers. Using RAPD markers. Agric. Biol, 8, 198–202.

Fan, J., Chen, K., Amombo, E., Hu, Z., Chen, L., & Fu, J. (2015). Physiological and Molecular Mechanism of Nitric Oxide (NO) Involved in Bermudagrass Response to Cold Stress. PLOS ONE, 10(7), e0132991. https://doi.org/10.1371/journal.pone.0132991

Fan, J., Ren, J., Zhu, W., Amombo, E., Fu, J., & Chen, L. (2014). Antioxidant Responses and Gene Expression in Bermudagrass under Cold Stress. Journal of the American Society for Horticultural Science, 139(6), 699–705. https://doi.org/10.21273/JASHS.139.6.699

Fernandez, O. N. (2003). Establishment of Cynodon dactylon from stolon and rhizome fragments. Weed Research, 43(2), 130–138. https://doi.org/10.1046/j.1365-3180.2003.00324.x

García, C., Guichoux, E., & Hampe, A. (2018). A comparative analysis between SNPs and SSRs to investigate genetic variation in a juniper species (Juniperus phoenicea ssp. Turbinata). Tree Genetics & Genomes, 14(6), 87. https://doi.org/10.1007/s11295-018-1301-x

Godwin, C., Fang, T., & Wu, Y. (2020). Genetic identity and diversity among experimental selections and cultivars of vegetatively propagated turf bermudagrass as assessed with SSR markers. International Turfgrass Society Research Journal, 14(1), 632–641. https://doi.org/10.1002/its2.29

Grossman, A. Y., Andrade, M. H. M. L., Chaves, A. L. A., Mendes Ferreira, M. T., Techio, V. H., Lopez, Y., Begcy, K., Kenworthy, K. E., & Rios, E. F. (2021). Ploidy Level and Genetic Parameters for Phenotypic Traits in Bermudagrass (Cynodon spp.) Germplasm. Agronomy, 11(5), 912. https://doi.org/10.3390/agronomy11050912

Hanna, W., Raymer, P., & Schwartz, B. (2013). Warm-Season Grasses: Biology and Breeding. In Turfgrass: Biology, Use, and Management (pp. 543–590). https://doi.org/10.2134/agronmonogr56.c16

Harlan, J. R. (1970). Cynodon species and their value for grazing and hay. Herbage Abstracts, 40, 233–238.

Harlan, J. R., & de Wet, J. M. J. (1969). Sources of Variation in Cynodon dactylon (L). Pers.1. Crop Science, 9(6), cropsci1969.0011183X000900060031x. https://doi.org/10.2135/cropsci1969.0011183X000900060031x

Harlan, J. R., de Wet, J. M. J., Huffine, W. W., & Deakin, J. R. (1970). A guide to the species of Cynodon (Gramineae). Bulletin of the Oklahoma Agricultural Experiment Station, B-673.

Harlan, J. R., de Wet, J. M. J., Rawal, K. M., Felder, M. R., & Richardson, W. L. (1970). Cytogenetic Studies in Cynodon L. C. Rich. (Gramineae). Crop Science, 10(3), cropsci1970.0011183X001000030023x. https://doi.org/10.2135/cropsci1970.0011183X001000030023x

Harris-Shultz, K. R., Schwartz, B. M., & Brady, J. A. (2011). Identification of Simple Sequence Repeat Markers that Differentiate Bermudagrass Cultivars Derived from 'Tifgreen.' Journal of the American Society for Horticultural Science, 136(3), 211–218. https://doi.org/10.21273/JASHS.136.3.211

Hu, L., Wang, Z., & Huang, B. (2009). Photosynthetic Responses of Bermudagrass to Drought Stress Associated with Stomatal and Metabolic Limitations. Crop Science, 49(5), 1902–1909. https://doi.org/10.2135/cropsci2008.12.0697

Hu, Z., Amombo, E., Gitau, M. M., Bi, A., Zhu, H., Zhang, L., Chen, L., & Fu, J. (2017). Changes of Antioxidant Defense System and Fatty Acid Composition in Bermudagrass under Chilling Stress. Journal of the American Society for Horticultural Science, 142(2), 101–109. https://doi.org/10.21273/JASHS03999-16

Hu, Z., Liu, A., Gitau, M. M., Huang, X., Chen, L., & Fu, J. (2018). Insights into the MicroRNA-regulated response of bermudagrass to cold and salt stress. Environmental and Experimental Botany, 145, 64–74. https://doi.org/10.1016/j.envexpbot.2017.10.026

Huang, C., Liu, G., Bai, C., & Wang, W. (2014). Genetic Analysis of 430 Chinese Cynodon dactylon Accessions Using Sequence-Related Amplified Polymorphism Markers. International Journal of Molecular Sciences, 15(10), Article 10. https://doi.org/10.3390/ijms151019134

Huang, C. Q., Huang, D. Y., Zhang, Y. F., & Liu, G. D. (2010). Genetic analysis for 57 accessions of Cynodon dactylon from 17 countries in 5 continents by SRAP markers. Trop Grasslands, 44, 274–281.

Huang, S., Jiang, S., Liang, J., Chen, M., & Shi, Y. (2019). Current knowledge of bermudagrass responses to abiotic stresses. Breeding Science, 69(2), 215–226. https://doi.org/10.1270/jsbbs.18164

Huffine, W. W. (1957). Sunturf Bermuda—A New Grass for Oklahoma Lawns: Vol. B-494 (pp. 1–7). Oklahoma Agricultural Experiment Station.

Hurcombe, R. E. (1948). A cytological and morphological study of cultivated Cynodon species. In Better turf through research (pp. 36–47).

Jewell, M. C., Zhou, Yi., Loch, D. S., Godwin, I. D., & Lambrides, C. J. (2012). Maximizing Genetic, Morphological, and Geographic Diversity in a Core Collection of Australian Bermudagrass. Crop Science, 52(2), 879–889. https://doi.org/10.2135/cropsci2011.09.0497

Jung, J., Park, S.-W., Liu, W. Y., & Kang, B.-C. (2010). Discovery of single nucleotide polymorphism in Capsicum and SNP markers for cultivar identification. Euphytica, 175(1), 91–107. https://doi.org/10.1007/s10681-010-0191-2

Kang, S.-Y., Lee, G.-J., Lim, K. B., Lee, H. J., Park, I. S., Chung, S. J., Kim, J.-B., Kim, D. S., & Rhee, H. K. (2008). Genetic Diversity among Korean Bermudagrass (Cynodon spp.) Ecotypes Characterized by Morphological, Cytological and Molecular Approaches. Molecules and Cells, 25(2), 163–171. https://doi.org/10.1016/S1016-8478(23)17566-2

Karaca, M., Lang, D. J., Yerk-Davis, G. L., Sukumar, S., & Ainsworth, A. J. (2000). Determination of DNA content and genome in Cynodon species by flow cytometry. Crop Research (Hisar), 20(1), 1–12.

Katuwal, K. B., Jespersen, D., Bhattarai, U., Chandra, A., Kenworthy, K. E., Milla-Lewis, S. R., Schwartz, B. M., Wu, Y., & Raymer, P. (2022). Multilocational screening identifies new drought-tolerant, warm-season turfgrasses. Crop Science, 62(4), 1614–1630. https://doi.org/10.1002/csc2.20726

Kim, C., Lemke, C., & Paterson, A. H. (2009). Functional dissection of drought-responsive gene expression patterns in Cynodon dactylon L. Plant Molecular Biology, 70(1), 1–16. https://doi.org/10.1007/s11103-009-9453-6

Li, Z., Huang, C., Zhao, J., Yu, G., Huang, T., Peng, Y., & Hassan, M. J. (2023). A bermudagrass variant exhibits strong tolerance to low temperature associated with enhanced sugar metabolism and cold-responsive pathways. Crop Science, 63(4), 2553–2568. https://doi.org/10.1002/csc2.20992

Ling, Y., Huang, L. K., Zhang, X. Q., Ma, X., Liu, W., Chen, S. Y., & Yan, H. D. (2015). Assessment of genetic diversity of bermudagrass germplasm from southwest China and Africa by using AFLP markers. Genetics and Molecular Research, 14(1), 1748–1756. https://doi.org/10.4238/2015.March.13.1

Morris, K. N., & Shearman, R. C. (1998). NTEP turfgrass evaluation guidelines. NTEP Turfgrass Evaluation Workshop, Beltsville, MD, 1–5.

Ndjiondjop, M. N., Semagn, K., Zhang, J., Gouda, A. C., Kpeki, S. B., Goungoulou, A., Wambugu, P., Dramé, K. N., Bimpong, I. K., & Zhao, D. (2018). Development of species diagnostic SNP markers for quality control genotyping in four rice (Oryza L.) species. Molecular Breeding, 38(11), 131. https://doi.org/10.1007/s11032-018-0885-z

Pembleton, L. W., Drayton, M. C., Bain, M., Baillie, R. C., Inch, C., Spangenberg, G. C., Wang, J., Forster, J. W., & Cogan, N. O. I. (2016). Targeted genotyping-by-sequencing permits cost-effective identification and discrimination of pasture grass species and cultivars. Theoretical and Applied Genetics, 129(5), 991–1005. https://doi.org/10.1007/s00122-016-2678-2

Pudzianowska, M., & Baird, J. H. (2021). Genetic diversity and species-specific DNA markers of Cynodon. Crop Science, csc2.20369. https://doi.org/10.1002/csc2.20369

Qian, Y. L., Fry, J. D., & Upham, W. S. (1997). Rooting and Drought Avoidance of Warm-Season Turfgrasses and Tall Fescue in Kansas. Crop Science, 37(3), cropsci1997.0011183X003700030034x. https://doi.org/10.2135/cropsci1997.0011183X003700030034x

Shi, H., Wang, Y., Cheng, Z., Ye, T., & Chan, Z. (2012). Analysis of Natural Variation in Bermudagrass (Cynodon dactylon) Reveals Physiological Responses Underlying Drought Tolerance. PLOS ONE, 7(12), e53422. https://doi.org/10.1371/journal.pone.0053422

Singh, L., Wu, Y., McCurdy, J. D., Stewart, B. R., Warburton, M. L., Baldwin, B. S., & Dong, H. (2023). Genetic diversity and population structure of bermudagrass (Cynodon spp.) revealed by genotyping-by-sequencing. Frontiers in Plant Science, 14. https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2023.1155721

Sliwinska, E. (2018). Flow cytometry – a modern method for exploring genome size and nuclear DNA synthesis in horticultural and medicinal plant species. Folia Horticulturae, 30(1), 103–128. https://doi.org/10.2478/fhort-2018-0011

Taliaferro, C. M. (2003). Bermudagrass (Cynodon (L.) Rich). In Turfgrass Biology, Genetics, and Breeding (pp. 235–256). John Wiley & Sons.

Taliaferro, C. M., Hopkins, A. A., Henthorn, J. C., Murphy, C. D., & Edwards, R. M. (1997). Use of flow cytometry to estimate ploidy level in Cynodon species. International Turfgrass Society Research Journal, 8, 385–392.

Thomashow, M. F. (2001). So What's New in the Field of Plant Cold Acclimation? Lots! Plant Physiology, 125(1), 89–93. https://doi.org/10.1104/pp.125.1.89

Tomaszewska, P., Pellny, T. K., Hernández, L. M., Mitchell, R. A. C., Castiblanco, V., de Vega, J. J., Schwarzacher, T., & Heslop-Harrison, P. (J S.). (2021). Flow Cytometry-Based Determination of Ploidy from Dried Leaf Specimens in Genomically Complex Collections of the Tropical Forage Grass Urochloa s. L. Genes, 12(7), Article 7. https://doi.org/10.3390/genes12070957

Vieira, M. L. C., Santini, L., Diniz, A. L., & Munhoz, C. de F. (2016). Microsatellite markers: What they mean and why they are so useful. Genetics and Molecular Biology, 39(3), 312–328. https://doi.org/10.1590/1678-4685-GMB-2016-0027

Wang, Z., Wu, Y., Martin, D. L., Gao, H., Samuels, T., & Tan, C. (2010). Identification of Vegetatively Propagated Turf Bermudagrass Cultivars Using Simple Sequence Repeat Markers. Crop Science, 50(5), 2103–2111. https://doi.org/10.2135/cropsci2010.02.0116

Wu, Y. Q., Taliaferro, C. M., Bai, G. H., & Anderson, M. P. (2004). AFLP analysis of Cynodon dactylon (L.) Pers. Var. Dactylon genetic variation. Genome, 47(4), 689–696. https://doi.org/10.1139/g04-032

Wu, Y. Q., Taliaferro, C. M., Bai, G. H., Martin, D. L., Anderson, J. A., Anderson, M. P., & Edwards, R. M. (2006). Genetic Analyses of Chinese Cynodon Accessions by Flow Cytometry and AFLP Markers. Crop Science, 46(2), 917–926. https://doi.org/10.2135/cropsci2005.08.0256

Yang, L., Wu, Y., Moss, J. Q., Zhong, S., & Yang, B. (2018). Molecular Identification and Characterization of Seeded Turf Bermudagrass Cultivars Using Simple Sequence Repeat Markers. Agronomy Journal, 110(6), 2142–2150. https://doi.org/10.2134/agronj2018.01.0068

Yoo, E., Haile, M., Ko, H.-C., Choi, Y.-M., Cho, G.-T., Woo, H.-J., Wang, X., Sung, P., Lee, J., Lee, J., & Ro, N. (2023). Development of SNP markers for Cucurbita species discrimination. Scientia Horticulturae, 318, 112089. https://doi.org/10.1016/j.scienta.2023.112089

Zhang, J., Wang, M., Guo, Z., Guan, Y., Guo, Y., & Yan, X. (2019). Variation in ploidy level and genome size of Cynodon dactylon (L.) Pers. Along a latitudinal gradient. Folia Geobotanica, 54(3/4), 267–278.

Zhang, J., Wang, M., Guo, Z., Guan, Y., Liu, J., Yan, X., & Guo, Y. (2019). Genetic Diversity and Population Structure of Bermudagrass [Cynodon dactylon (L.) Pers.] along Latitudinal Gradients and the Relationship with Polyploidy Level. Diversity, 11(8), Article 8. https://doi.org/10.3390/d11080135

Zhang, J., Yang, J., Lv, Y., Zhang, X., Xia, C., Zhao, H., & Wen, C. (2023). Genetic diversity analysis and variety identification using SSR and SNP markers in melon. BMC Plant Biology, 23(1), Article 1. https://doi.org/10.1186/s12870-023-04056-7

Zhang, X., Ervin, E. h., Waltz, C., & Murphy, T. (2011). Metabolic Changes During Cold Acclimation and Deacclimation in Five Bermudagrass Varieties: II. Cytokinin and Abscisic Acid Metabolism. Crop Science, 51(2), 847–853. https://doi.org/10.2135/cropsci2010.06.0346

Zhang, X., Wang, K., Ervin, E. H., Waltz, C., & Murphy, T. (2011). Metabolic Changes During Cold Acclimation and Deacclimation in Five Bermudagrass Varieties. I. Proline, Total Amino Acid, Protein, and Dehydrin Expression. Crop Science, 51(2), 838–846. https://doi.org/10.2135/cropsci2010.06.0345

Zhou, P., An, Y., Wang, Z., Du, H., & Huang, B. (2014). Characterization of Gene Expression Associated with Drought Avoidance and Tolerance Traits in a Perennial Grass Species. PLOS ONE, 9(8), e103611. https://doi.org/10.1371/journal.pone.0103611

Zhou, Y., Lambrides, C. J., & Fukai, S. (2013). Drought Resistance of C4 Grasses Under Field Conditions: Genetic Variation Among a Large Number of Bermudagrass (Cynodon spp.) Ecotypes Collected from Different Climatic Zones. Journal of Agronomy and Crop Science, 199(4), 253–263. https://doi.org/10.1111/jac.12020

Zhu, H., Yu, X., Xu, T., Wang, T., Du, L., Ren, G., & Dong, K. (2015). Transcriptome profiling of cold acclimation in bermudagrass (Cynodon dactylon). Scientia Horticulturae, 194, 230–236. https://doi.org/10.1016/j.scienta.2015.08.024

Chapter 1

What's in a name? Exploring the systematics of bermudagrass (Cynodon spp.)

Abstract

Species misclassification has been reported in previous genetic diversity studies of bermudagrass (*Cynodon* spp.) as well as numerous other plant species. Traditional taxonomic classifications relying on visual assessment of morphological traits worked reasonably well when other approaches were unavailable. With current approaches offering more objective methods, old classifications can be revised and made more precise. One of such approaches is the use of DNA markers. This study attempted to reassess the species assignments of accessions in the bermudagrass germplasm collection at the University of California, Riverside using a combination of data offering a more objective approach to species classifications. Based on cytological, morphological, and DNA marker data, 30 of 125 accessions (24%) in this study were recommended for reclassification. Cytological data alone identified 28 (22%) triploid accessions, prompting their removal from future breeding efforts and a discussion on whether triploid accessions should be considered as species (as is the case with *Cynodon* × *magennisii*). Additionally, an exploratory analysis was performed in an attempt to identify species-specific DNA markers, and 211 such single-locus markers were found across the analyzed accessions. Using the Kompetitive Allele-Specific PCR, 46 of these markers were used to genotype the

germplasm collection, with four showing promising results for discrimination among *C. barberi, C. plectostachyus,* and *C. transvaalensis* accessions. These results show a way to develop more effective diagnostic tools for species discrimination, which would benefit programs targeting the minor *Cynodon* taxa (*C. aethiopicus, C. barberi, C. incompletus, C. nlemfluensis, C. plectostachyus, C. radiatus*).

Introduction

The classification of living organisms into groups has existed for thousands of years, with early groupings based mainly on morphological similarities and differences. The word “taxonomy” was first coined in the early nineteenth century by botanist Augustin Pyramus de Candolle to describe the scientific discipline of classifying plants (De Candolle and de Candolle 1844), though it was later revised to be inclusive of all organisms (Heywood and Watson 1995). Early classifications were functional, both in their methods and in their applications to organisms of value, such as plants with medicinal properties, and lacked any attempts at hierarchical classification (Raven 2004). Over time, the discipline evolved to include other traits and characters as a means of distinction, such as morphology, cytology, genetics, and biochemistry, and focused on character constancy for groupings (Haider 2018; Rouhan and Gaudeul 2014). Although some of these methods may be objective by nature, classification schemes are often subjective and at the discretion of the taxonomist. Throughout

history, it has not been uncommon to find conflicting views between taxonomists, especially in grass species with intermediate ecotypes. A typical example is a group of species of bermudagrasses (Hurcombe 1948; de Wet and Harlan 1970). This eventually led to more modern taxonomic methods and concepts that are focused on quantitative analyses or evolutionary relationships, such as numerical taxonomy (Sokal and Sneath 1963) or cladistic theory (Hennig 1999). Genetic markers and high-throughput sequencing have also played a role in advancing the field of taxonomy. Phylogenetic studies have enabled researchers to distinguish and group individuals based on their genetic relatedness. Recent studies have utilized the wealth of information derived from genetic sequencing of both plastid and nuclear genomes and have used methods such as maximum likelihood and Bayesian analyses to better assign species groupings (Hilu, 2007; Peterson et al., 2010).

Members of the genus *Cynodon* (family *Poaceae*, subfamily *Chloridoideae*, tribe *Cynodonteae,* subtribe *Chloridinae*), often collectively referred to as bermudagrass, are warm-season perennial grasses that display a wide range of morphologies, making them suitable for use as turfgrasses or forage crops. As a warm-season grass, bermudagrass exhibits high heat and drought tolerance, and is commonly found in semi-arid and arid regions of the world. Although many *Cynodon* species are endemic to regions such as East Africa, South Africa, and South Asia, bermudagrass are present all over the world and in regions normally considered outside of its natural range (Assefa et

al. 1999; Hanna, Raymer, and Schwartz 2013; Taliaferro 2003). Differences in morphological adaptations can be observed among the species and their geographic distributions. There are nine commonly listed species of bermudagrass: *Cynodon aethiopicus* Clayton et Harlan, *C. arcuatus* J. S. Presl ex C. B. Presl (now seen as a heterotypic synonym for *C. radiatus* Roth), *C. barberi* Rang. et Tad., *C. dactylon* (L.) Pers., *C. incompletus* Nees, *C. nlemfuensis* Vanderyst, *C. plectostachyus* (K. Schum.) Pilg., *C. transvaalensis* Burtt-Davy, and *C.* × *magennisii* Hurcombe (Harlan et al. 1970). Of the nine species, *C. dactylon* and *C. transvaalensis* are regarded as the two major taxa within the genus for their ability to function as turfgrasses. The remaining seven taxa are considered minor, and they vary in their usefulness as turfgrass or forage crops. Compatibility with one or both of the major taxa can enhance the value of these minor taxa as a source of genetic diversity for breeding programs (Hanna et al. 2013; Taliaferro 2003). Diploid *C. aethiopicus* are capable of producing hybrids with *C. nlemfuensis,* while tetraploid *C. aethiopicus* can hybridize with *C. dactylon*. Diploid *C. nlemfuensis* is compatible with *C. transvaalensis*. *C. incompletus* is compatible with *C. dactylon* (de Wet and Harlan 1970). *C. acruatus*, *C. barberi*, and *C. plectostachyus* have not been reported to be compatible with either *C. dactylon* or *C. transvaalensis*.

Bermudagrass species are prime examples of how phenotypic variability can pose a challenge for taxonomic classifications. Current *Cynodon* species assignments follow the guidelines set by de Wet and Harlan in 1970 after their

extensive revision of the *Cynodon* systematics. Their classification of the genus was based on morphology, geographic distribution, ecological behavior, cytogenetic data, and sexual compatibility (de Wet and Harlan 1970), and although characteristics have been defined for each of the species, intermediate ecotypes with morphological similarities have been observed both within and between species. This range in phenotypic variation has proven to be challenging for *Cynodon* taxonomic classifications, and current classifications by de Wet and Harlan can be viewed as an attempt to strike a balance between phenetic and phylogenetic methods, highlighting the need for more quantitative methods. Differences between species assignments by collectors and phylogenetic groupings have been observed in several genetic diversity studies of the *Cynodon* genus (Assefa et al. 1999; Jewell et al. 2012; Pudzianowska and Baird 2021). These studies had similar results, with species described as 'genetically isolated' by de Wet and Harlan grouped closer than expected to other species viewed as more distant. Assefa et al. (1999) also noted that groupings based on genetic markers did not correspond well with known hybridization potentials among the taxa. Both Jewell et al. (2012) and Pudzianowska and Baird (2021) identified potential hybrids among the accessions studied, providing an opportunity for removing redundant accessions from germplasm collections. Pudzianowska and Baird (2021) also noted the possibility of misclassifying or mislabeling certain accessions when relying solely on morphological characters,

thus emphasizing the need for other metrics such as cytogenetics and DNA markers to support species classifications.

In this study, the taxonomic classifications of one hundred twenty-five bermudagrass accessions comprised of seven *Cynodon* species from the University of California, Riverside bermudagrass germplasm collection were reassessed using a combination of cytogenetic, morphological, geographic, and genetic marker data. Most studied accessions originated from the USDA collection. Reexamination of species classifications based on recent findings suggested some cases of misclassification or mislabeling. DNA data in the form of single nucleotide polymorphisms (SNP) and silicoDArT markers previously generated using the Diversity Arrays Technology sequencing (DArTseq) platform by Pudzianowska and Baird (2021) and reanalyzed herein. DArTseq is a high-throughput next-generation sequencing (NGS) method that is becoming increasingly popular in phylogenetic and genetic diversity studies for its ability to generate thousands of genetic markers without the need for prior DNA sequence knowledge (Kilian et al. 2012; Sansaloni et al. 2011), making it the ideal genotyping method for crops lacking a reference genome, such as bermudagrass. The objective of this study was to reexamine accessions in our germplasm collection for discrepancies in their species listings to make more informed decisions for future breeding efforts. By utilizing multiple types of data, this work attempts to clarify the observed inconsistencies in species assignments of bermudagrass accessions. Furthermore, we perform an exploratory study into

developing species-specific genetic markers that may function as a tool for distinguishing between *Cynodon* species.

Methods

Plant material

One hundred twenty-five accessions were selected from the bermudagrass germplasm collection maintained at the University of California, Riverside (UCR). The collection consists of accessions obtained from the United States Department of Agriculture– Agricultural Research Service National Plant Germplasm System (USDA-ARS NPGS) as well as material donated by Dr. Jeff Krans (Professor Emeritus, Mississippi State University). The collection includes accessions from seven of the *Cynodon* species from originating from different geographical regions and has been previously described (Pudzianowska and Baird 2021). Briefly, inconsistencies were found for multiple accessions between their listed species identity and their genetic grouping as determined by genetic markers. To verify the potential sources of error (i.e. mislabeling during handling and transplanting events, contamination from unchecked plant growth or from spontaneous hybridization), questionable accessions were re-ordered and obtained from the USDA-ARS NPGS. Accessions that showed similar morphological appearance and consistent grouping (original and reordered) were retained in the study. The list of accessions used in this study includes those

used by Pudzianowska and Baird (2021) and additional accessions from the UCR bermudagrass germplasm collection (Table 1.1).

Chromosome counts

Specimen were placed in an aerated hydroponic solution for several days, and actively growing roots were collected to ice water, treated for ca. 24 hours and fixed in a freshly prepared mixture of 3 parts absolute ethanol to one-part glacial acetic acid. Fixed roots were stained in 2% acetocarmine for at least 2 h. Tips of the roots were cut off with a razor blade, placed onto a glass microscope slide in a drop of 45% acetic acid and squashed under a cover slip by gentle tapping with the blunt end of a wooden toothpick. Such preparations were heated gently over an open flame and pressed from above to flatten the cells. Preparations were examined under a ZEISS Axioskop (Carl Zeiss NTS Ltd., Oberkochen, Germany) microscope using the 10x, 40x, and 100x (oil) objectives. Chromosomes were counted visually and, when possible, images of chromosomes were captured using a digital camera.

Table 1.1 List of *Cynodon* accessions with their source, listed species, and country of origin. Includes accessions previously listed by Pudzianowska and Baird (2021).

Accession	Source	Species	Country of origin	Accession	Source	Species	Country of origin
B1	J. Krans	C. transvaalensis	N/A[a]	B36	PI 647878	C. transvaalensis	Australia
B2	J. Krans	C. transvaalensis	N/A	B37	PI 615161	C. transvaalensis	Israel
B3	J. Krans	C. transvaalensis	N/A	B38	PI 647879	C. transvaalensis	Australia
B4	J. Krans	C. transvaalensis	N/A	B39	PI 647880	C. transvaalensis	Australia
B5	J. Krans	C. incompletus	N/A	B40	PI 647881	C. transvaalensis	Australia
B6	J. Krans	C. incompletus	N/A	B42	PI 414700	C. plectostachyus	South Africa
B7	J. Krans	C. incompletus	N/A	B43	PI 414704	C. plectostachyus	South Africa
B8	J. Krans	C. barberi	N/A	B44	PI 267985	C. dactylon	Pakistan
B9	J. Krans	C. barberi	N/A	B45	PI 290880	C. dactylon	South Africa
B10	J. Krans	C. transvaalensis	N/A	B46	PI 287256	C. dactylon	Sri Lanka
B11	J. Krans	C. barberi	N/A	B48	PI 295339	C. dactylon	Germany
B12	J. Krans	C. barberi	N/A	B49	PI 292142	C. dactylon	Ghana
B13	J. Krans	C. transvaalensis	N/A	B50	PI 225956	C. dactylon	Ethiopia
B14	J. Krans	C. barberi	N/A	B51	PI 292250	C. dactylon	Philippines
B16	J. Krans	C. transvaalensis	N/A	B52		C. dactylon	N/A
B17	J. Krans	C. transvaalensis	N/A	B53	PI 292508	C. dactylon	Japan
B18	J. Krans	C. incompletus	N/A	B54	PI 255447	C. dactylon	Kenya
B19	J. Krans	C. barberi	N/A	B55	PI 223249	C. dactylon	Afghanistan
B20	J. Krans	C. transvaalensis	N/A	B56	PI 204438	C. dactylon	Turkey
B21	J. Krans	C. barberi	N/A	B57	PI 251108	C. dactylon	Italy
B24	PI 595197	C. aethiopicus	Ethiopia	B59	PI 288221	C. dactylon	Madagascar
B25	PI 292570	C. radiatus	Philippines	B60	PI 287139	C. barberi	India
B26	PI 291190	C. incompletus	South Africa	B61	PI 287145	C. barberi	India
B27	PI 291620	C. incompletus	South Africa	B62	PI 287143	C. barberi	India
B28	PI 291753	C. dactylon	South Africa	B63	PI 287142	C. barberi	India
B30	PI 299181	C. transvaalensis	Ethiopia	B65	PI 287255	C. barberi	Sri Lanka
B31	PI 290894	C. transvaalensis	South Africa	B66	PI 287253	C. barberi	Sri Lanka
B32	PI 286584	C. transvaalensis	India	B67	PI 288042	C. barberi	India
B33	PI 290813	C. transvaalensis	South Africa	B68a		C. dactylon	N/A
B34	PI 289923	C. transvaalensis	South Africa	B68b		C. dactylon	N/A
B35	PI 491560	C. transvaalensis	South Africa	B68c		C. dactylon	N/A

[a] N/A, not applicable.

Table 1.1 (Continued)

Accession	Source	Species	Country of origin	Accession	Source	Species	Country of origin
B91	PI 224149	C. dactylon	South Africa	B126	PI 564242	Cynodon spp.	Zimbabwe
B92	PI 224694	C. dactylon	Zambia	B127	PI 290891	C. incompletus	South Africa
B93	PI 225591	C. dactylon	Tanzania	B128	PI 291597	Cynodon spp.	Zimbabwe
B94	PI 287147	C. dactylon	India	B129	PI 291755	Cynodon spp.	South Africa
B95	PI 287149	C. dactylon	India	B131	PI 290872	C. transvaalensis	South Africa
B96	PI 287151	C. dactylon	India	B132	PI 290905	C. transvaalensis	South Africa
B97	PI 287154	C. dactylon	India	B133	PI 491560	C. dactylon	South Africa
B98	PI 287155	C. dactylon	India	B134		C. aethiopicus	Ethiopia
B99	PI 287157	C. dactylon	India	B135	PI 287143	C. barberi	India
B100	PI 287244	C. dactylon	India	B136	PI 287254	C. barberi	Sri Lanka
B101	PI 287246	C. dactylon	India	B137	PI 288042	C. barberi	India
B102	PI 287247	C. dactylon	India	B138	PI 641703	C. dactylon	China
B103	PI 288043	C. dactylon	India	B139	PI 297827	C. dactylon	Israel
B104	PI 288216	C. dactylon	Madagascar	B140	PI 288221	C. dactylon	Madagascar
B105	PI 288676	C. dactylon	Madagascar	B141	PI 289714	C. dactylon	Madagascar
B106	PI 289913	C. dactylon	South Africa	B142	PI 223249	C. dactylon	Afghanistan
B107	PI 289930	C. dactylon	South Africa	B143	PI 225956	C. dactylon	Ethiopia
B108	PI 290656	C. dactylon	South Africa	B144	PI 251108	C. dactylon	Macedonia
B109	PI 290657	C. dactylon	South Africa	B145	PI 255447	C. dactylon	Kenya
B110	PI 290667	C. dactylon	South Africa	B146	PI 287256	C. dactylon	Sri Lanka
B111	PI 290881	C. dactylon	South Africa	B147	PI 288676	C. dactylon	Madagascar
B112	PI 290883	C. dactylon	South Africa	B148	PI 290880	C. dactylon	South Africa
B113	PI 290887	C. dactylon	South Africa	B149	PI 292142	C. dactylon	Ghana
B114	PI 290991	C. dactylon	South Africa	B151	PI 292508	C. dactylon	Japan, Honshu
B116	PI 291155	C. dactylon	South Africa	B152	PI 295339	C. dactylon	Germany
B117	PI 291164	C. dactylon	South Africa	B156	PI 289613	C. radiatus	Madagascar
B119	PI 291586	C. dactylon	Zimbabwe	B158	PI 289923	C. transvaalensis	South Africa
B120	PI 291726	C. dactylon	South Africa	B159	PI 290813	C. transvaalensis	South Africa
B121	PI 291966	C. dactylon	Kenya	B160	PI 291981	C. transvaalensis	Ethiopia
B123	PI 364488	C. dactylon	South Africa	B162	PI 647878	C. transvaalensis	Australia
B124	PI 564237	C. dactylon	Australia	B163	PI 647879	C. transvaalensis	Australia
B125	PI 564240	C. dactylon	Zimbabwe				

Flow cytometry

Three leaves, approximately 2-3 cm in length each, were collected from each accession and placed into a 1.5 mL microcentrifuge tube with a drop of water to retain moisture. Samples were sent to the Centre of Plant Structural and Functional Genomics at the Institute of Experimental Botany of the Czech Academy of Sciences (Olomouc, Czech Republic) for DNA content analysis. Soybean (2C = 2.5 pg) was used as the internal standard for each sample, and several samples were selected at random to be reanalyzed for reproducibility.

Morphology

Morphological characteristics of each bermudagrass accession were visually examined to allow for comparisons between accessions. Each bermudagrass accession was grown and maintained under greenhouse conditions in 2-gallon pots filled with soil (UC Soil Mix II). Morphological characteristics were observed after several weeks without clipping to allow for traits to develop and become more distinguishable. These characteristics included leaf length, leaf width, leaf color, the presence of hairs, growth pattern, and density. Digital images were taken to capture both the side and top profiles of each plant.

DNA marker analysis

Each accession was genotyped using DArTseq (Kilian et al. 2012) as described by Pudzianowska and Baird (2021). For this study, the original genotype data were re-analyzed. The DArTseq data was processed using the dartR package (Gruber et al. 2018) in R 3.6.3 (R Core Team 2020). Data were filtered by removing 1) markers with the reproducibility below 100%, 2) markers that were monomorphic, and 3) markers that had a call rate less than 95%. SilicoDArT markers were additionally filtered to remove those with a polymorphic information content (PIC) below 0.4. Rather than filtering by PIC, SNP markers were filtered for secondary SNP markers (markers that share a sequence tag). Distance matrices were calculated using the Euclidean distance method based on the presence-absence data (silicoDArT) or allele frequencies (SNP). These matrices were then used to build unrooted-dendrograms based on the unweighted pair group method (UPGMA) using a hierarchical clustering function, hclust, available in R 3.6.3. A Mantel test was performed with 9,999 permutations to calculate the correlation between the silicoDArT- and SNP-based distance matrices using the ade4 package (Dray and Dufour 2007). The genetic structure was analyzed for the silicoDArT and SNP markers using STRUCTURE 2.3.4 (Pritchard, Stephens, and Donnelly 2000), with five runs for each value of *K* from 2 to 9 using a burn-in period of 10,000 and 20,000 Markov chain Monte Carlo (MCMC) iterations. Both the admixture model and correlated allele frequencies were assumed. Structure Harvester (Earl and vonHoldt 2012) was used to

determine the most probable number of clusters (*K*) using the Evanno method. After determining the most likely *K*-value, an additional run was performed for the silicoDArT and SNP data using the same parameters (i.e. admixture and correlated frequencies assumed), but higher burn-in and MCMC iterations (100,000 and 200,000, respectively). The use of STRUCTURE and Structure Harvester, as well as cluster visualization, were facilitated by the program Structure_threader (Pina-Martins et al. 2017) to parallelize and automate the analyses. Principal component analysis (PCA) was performed and visualized in dartR. Scatter plots were used to visualize clustering of accessions and to confirm results from Structure Harvester.

Reanalysis of the genotyping data was performed iteratively to incorporate the cytological and morphological data needed for suggesting new species assignments. The data was reanalyzed multiple times using different subpopulations of the original one hundred twenty-five accessions (data not shown). Subpopulations were created based on genetic, cytological, and morphological factors. Individual accessions which did not group with their listed species based on genetic analysis (DArTseq) were deemed as candidates to be 'reassigned' to the inferred species listing that best fit the accession. This was then confirmed with cytological data; the listed species name for each accession had to match their counted ploidy level. For example, if an accession was found to be tetraploid, it could not be a *C. transvaalensis*; *C. transvaalensis* is a diploid species. After filtering the accession list for duplicates and triploids, a

subpopulation of 86 accessions was obtained for population analysis and species-specific marker development. The accessions selected for this study, along with their original and suggested species listings, ploidy, 2C content, and morphological grouping, are listed in Table 1.2. If the inferred species matched the original listing, the column was left blank.

Unique SNP markers were identified by comparing the presence/absence of each marker on an accession- and species-wide basis. SNP markers were identified as ‘species-’, or ‘group-’, specific if they were present/absent only in individuals of a given group using a custom shell script. First, SNP markers were tested across all individuals of a given species. Markers present or absent in all individuals of a given species were selected, then compared against the selected markers for the other species. If a marker was found to be present or absent in only one species, then it was considered unique and therefore ‘species-’ or ‘group-’ specific. The identified species-specific markers were then used to develop primers for genotyping. Markers were aligned to a reference contig assembly of the bermudagrass cultivar, ‘Tifway’ (Hulse-Kemp, [unpublished data] 2022). ‘Tifway’ is a triploid hybrid between *C. transvaalensis* x *C. dactylon* developed in Tifton, GA, USA (Burton 1966). Alignments were performed using BLAST (Madden 2013) to ensure selected markers were unique in the genome. Markers that were not a perfect match or found to be multilocational were discarded. Using bedtools (Quinlan and Hall 2010), 200 bp upstream and 200 bp downstream of the final SNP marker were gathered for sequence context and

Kompetitive Allele Specific PCR (KASP) primer design. A total of 46 sequences were sent to LGC Biosearch Technologies (Hoddesdon, UK) for primer optimization with their KASP genotyping platform (Table 1.3).

DNA extraction and marker validation

The identified species-/group-specific markers were validated using KASP genotyping. This assay uses a form of competitive allele-specific PCR with a fluorescence-based reporting system to identify and measure the variability at the nucleotide level (He, Holme, and Anthony 2014; Kumpatla et al. 2012; Semagn et al. 2014). Genomic DNA was extracted from fresh leaf tissue from selected accessions using the NucleoMag 384 Plant Kit (MACHERY-NAGEL Inc., Pennsylvania, USA) according to the manufacturer's protocol. DNA concentrations were quantified using a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA), then transferred to a 96-well plate and sent to LGC Biosearch Technologies for KASP genotyping.

Table 1.2 List of *Cynodon* accessions with their original species listing, followed by their inferred species based on chromosome counts, flow cytometry, and visual inspection of morphological characters.

Accession	Listed Species	Inferred species	Ploidy	2C (pg)	Morphological grouping
B1	*C. transvaalensis*	*C. transvaalensis*	2	7.975	2
B2	*C. transvaalensis*	*C. barberi*	2	5.475	12
B3	*C. transvaalensis*	*C. dactylon*	4	4.525	N/A[a]
B4	*C. transvaalensis*	*C. transvaalensis*	2	8.325	2
B5	*C. incompletus*	*C. incompletus*	3	5.625	N/A
B6	*C. incompletus*	*C. dactylon*	2	5.775	8
B7	*C. incompletus*	*C. dactylon*	3	5.425	3
B8	*C. barberi*	*C. barberi*	3	5.650	N/A
B9	*C. barberi*	*C. barberi*	3	4.700	N/A
B10	*C. transvaalensis*	*C. transvaalensis*	2	7.975	2
B11	*C. barberi*	*C. dactylon*	4	4.625	N/A
B12	*C. barberi*	*C. barberi*	3	5.600	6
B13	*C. transvaalensis*	*C. transvaalensis*	2	8.125	3
B14	*C. barberi*	*C. barberi*	3	5.550	6
B16	*C. transvaalensis*	*C. transvaalensis*	2	8.575	3
B17	*C. transvaalensis*	*C. transvaalensis*	2	N/A	N/A
B18	*C. incompletus*	*C. incompletus*	2	N/A	N/A
B19	*C. barberi*	*C. barberi*	3	5.550	6
B20	*C. transvaalensis*	*C. barberi*	3	5.575	6
B21	*C. barberi*	*C. barberi*	3	5.425	11
B24	*C. aethiopicus*	*C. aethiopicus*	4	4.675	1
B25	*C. radiatus*	*C. radiatus*	3	N/A	N/A
B26	*C. incompletus*	*C. incompletus*	2	N/A	N/A
B27	*C. incompletus*	*C. incompletus*	4	4.525	N/A
B28	*C. dactylon*	*C. transvaalensis*	2	4.150	11
B30	*C. transvaalensis*	*C. transvaalensis*	2	7.850	2
B31	*C. transvaalensis*	*C. transvaalensis*	3	4.175	8
B32	*C. transvaalensis*	*C. dactylon*	3	5.475	N/A
B33	*C. transvaalensis*	*C. incompletus*	2	5.675	N/A
B34	*C. transvaalensis*	*C. transvaalensis*	3	4.425	5
B35	*C. transvaalensis*	*C. dactylon*	4	4.550	12
B36	*C. transvaalensis*	*C. dactylon*	4	4.700	N/A
B37	*C. transvaalensis*	*C. dactylon*	4	5.025	N/A
B38	*C. transvaalensis*	*C. dactylon*	4	4.575	N/A
B39	*C. transvaalensis*	*C. transvaalensis*	2	7.825	N/A
B40	*C. transvaalensis*	*C. transvaalensis*	2	7.950	3
B42	*C. plectostachyus*	*C. plectostachyus*	2	7.950	N/A
B43	*C. plectostachyus*	*C. plectostachyus*	2	7.975	4
B44	*C. dactylon*	*C. dactylon*	4	4.575	N/A
B45	*C. dactylon*	*C. dactylon*	4	5.575	5
B46	*C. dactylon*	*C. transvaalensis*	3	N/A	N/A
B48	*C. dactylon*	*C. dactylon*	3	5.525	3
B49	*C. dactylon*	*C. dactylon*	4	N/A	N/A
B50	*C. dactylon*	*C. transvaalensis*	2	7.925	3

[a] N/A, not applicable.

Table 1.2 (Continued)

Accession	Listed Species	Inferred species	Ploidy	2C (pg)	Morphological grouping
B51	*C. dactylon*	*C. dactylon*	3	N/A[a]	N/A
B52	*C. dactylon*	*C. transvaalensis*	2	8.025	2
B53	*C. dactylon*	*C. dactylon*	4	4.700	7
B54	*C. dactylon*	*C. dactylon*	3	5.400	9
B55	*C. dactylon*	*C. barberi*	3	5.625	6
B56	*C. dactylon*	*C. dactylon*	4	3.650	4
B57	*C. dactylon*	*C. dactylon*	N/A	N/A	N/A
B59	*C. dactylon*	*C. dactylon*	4	4.675	N/A
B60	*C. barberi*	*C. dactylon*	4	4.875	7
B61	*C. barberi*	*C. barberi*	2	5.500	12
B62	*C. barberi*	*C. transvaalensis*	2	N/A	N/A
B63	*C. barberi*	*C. barberi*	2	5.550	6
B65	*C. barberi*	*C. barberi*	2	5.625	6
B66	*C. barberi*	*C. barberi*	2	N/A	N/A
B67	*C. barberi*	*C. dactylon*	2	N/A	N/A
B68a	*C. dactylon*	*C. dactylon*	4	4.650	N/A
B68b	*C. dactylon*	*C. dactylon*	N/A	N/A	N/A
B68c	*C. dactylon*	*C. dactylon*	4	4.000	4
B91	*C. dactylon*	*C. dactylon*	4	4.275	N/A
B92	*C. dactylon*	*C. dactylon*	4	4.575	12
B93	*C. dactylon*	*C. dactylon*	N/A	N/A	N/A
B94	*C. dactylon*	*C. dactylon*	4	4.550	N/A
B95	*C. dactylon*	*C. dactylon*	4	4.525	N/A
B96	*C. dactylon*	*C. dactylon*	4	4.500	12
B97	*C. dactylon*	*C. dactylon*	2	5.350	N/A
B98	*C. dactylon*	*C. dactylon*	4	5.950	N/A
B99	*C. dactylon*	*C. dactylon*	4	4.475	11
B100	*C. dactylon*	*C. dactylon*	N/A	N/A	N/A
B101	*C. dactylon*	*C. dactylon*	4	4.800	N/A
B102	*C. dactylon*	*C. dactylon*	4	4.475	10
B103	*C. dactylon*	*C. dactylon*	N/A	5.375	N/A
B104	*C. dactylon*	*C. dactylon*	4	4.575	1
B105	*C. dactylon*	*C. transvaalensis*	2	7.475	2
B106	*C. dactylon*	*C. dactylon*	4	4.725	7
B107	*C. dactylon*	*C. dactylon*	4	5.225	2
B108	*C. dactylon*	*C. dactylon*	2	5.825	N/A
B109	*C. dactylon*	*C. dactylon*	3	5.850	7
B110	*C. dactylon*	*C. dactylon*	4	4.700	N/A
B111	*C. dactylon*	*C. dactylon*	N/A	N/A	N/A
B112	*C. dactylon*	*C. dactylon*	4	5.500	12
B113	*C. dactylon*	*C. dactylon*	N/A	4.625	N/A
B114	*C. dactylon*	*C. dactylon*	3	6.100	N/A
B116	*C. dactylon*	*C. dactylon*	4	4.850	N/A
B117	*C. dactylon*	*C. dactylon*	4	4.900	7

[a] N/A, not applicable.

Table 1.2 (Continued)

Accession	Listed Species	Inferred species	Ploidy	2C (pg)	Morphological grouping
B119	*C. dactylon*	*C. dactylon*	4	4.375	12
B120	*C. dactylon*	*C. dactylon*	4	4.925	7
B121	*C. dactylon*	*C. dactylon*	3	5.425	9
B123	*C. dactylon*	*C. dactylon*	N/A[a]	N/A	N/A
B124	*C. dactylon*	*C. dactylon*	3	4.450	11
B125	*C. dactylon*	*C. dactylon*	3	4.375	12
B126	*Cynodon spp.*	*C. transvaalensis*	2	8.275	2
B127	*C. incompletus*	*C. incompletus*	4	4.250	N/A
B128	*Cynodon spp.*	*C. dactylon*	3	5.050	N/A
B129	*Cynodon spp.*	*C. dactylon*	4	4.950	N/A
B131	*C. transvaalensis*	*C. dactylon*	4	4.350	12
B132	*C. transvaalensis*	*C. transvaalensis*	2	7.300	2
B133	*C. dactylon*	*C. transvaalensis*	2	3.950	2
B134	*C. aethiopicus*	*C. aethiopicus*	3	5.450	6
B135	*C. barberi*	*C. barberi*	2	5.575	6
B136	*C. barberi*	*C. barberi*	2	5.350	11
B137	*C. barberi*	*C. barberi*	2	5.550	6
B138	*C. dactylon*	*C. dactylon*	2	4.150	N/A
B139	*C. dactylon*	*C. dactylon*	2	7.325	N/A
B140	*C. dactylon*	*C. dactylon*	4	4.650	4
B141	*C. dactylon*	*C. dactylon*	4	4.600	4
B142	*C. dactylon*	*C. barberi*	2	5.575	6
B143	*C. dactylon*	*C. dactylon*	N/A	N/A	N/A
B144	*C. dactylon*	*C. dactylon*	3	4.500	10
B145	*C. dactylon*	*C. dactylon*	4	5.300	N/A
B146	*C. dactylon*	*C. barberi*	3	5.675	6
B147	*C. dactylon*	*C. transvaalensis*	2	7.425	2
B148	*C. dactylon*	*C. dactylon*	4	4.700	N/A
B149	*C. dactylon*	*C. dactylon*	4	4.350	N/A
B151	*C. dactylon*	*C. dactylon*	N/A	N/A	N/A
B152	*C. dactylon*	*C. dactylon*	2	4.475	N/A
B156	*C. radiatus*	*C. barberi*	2	5.550	6
B158	*C. transvaalensis*	*C. dactylon*	4	4.175	5
B159	*C. transvaalensis*	*C. transvaalensis*	3	4.175	5
B160	*C. transvaalensis*	*C. dactylon*	4	4.225	11
B162	*C. transvaalensis*	*C. dactylon*	4	4.625	N/A
B163	*C. transvaalensis*	*C. dactylon*	4	4.500	N/A

[a] N/A, not applicable.

Table 1.3 List of exploratory species-specific primers used for Kompetitive Allele-Specific (KASP) genotyping.

Primer ID	Allele X Primer	Allele Y Primer	Common Primer	Allele X	Allele Y
K0001	ACAACATCTAACAGGACGTCGC	ACAACATCTAACAGGACGTCGG	AGCTAGGCCAAGACTAGATCCCAAA	G	C
K0002	CATGACGACGTCGCCC	GCTCATGACGACGTCGCCG	CGTCCACAACCCCAACCGCTT	G	C
K0003	TCCCGGTAAGTGTTCTCTGC	CTTCCCGGTAAGTGTTCTCTGG	AAAGACCACTGCTGTGTCAGTCACT	G	C
K0004	CAGCTGCAGCACCTGCCG	CCAGCTGCAGCACCTGCCA	GGCAGGTGGCGCAGGTGCT	G	A
K0005	CACCGCAGCGTCTGCAGC	CCACCGCAGCGTCTGCAGT	ACGGCTCGCCGCGCGTGTT	C	T
K0006	AGTCCGGGACCCTCCTTCC	CAGTCCGGGACCCTCCTTCT	CAGGGCCTCTCGGGCCTGTT	G	A
K0007	CCACTACTAGCCGCACCCG	CCACTACTAGCCGCACCCC	GACATGGCCCGGTCGAGCAA	C	G
K0008	GCGCTGACGTCGCACGAG	GCGCTGACGTCGCACGAC	CCGAGAGCGTCTCCTGATGGAA	C	G
K0009	TCGACAAAGACCCACTGCGCT	CGACAAAGACCCACTGCGCC	GTAAGTGGCGAGCACCTCGTCAT	T	C
K0010	ACTGCCATTGCGACGTCGCAA	CTGCCATTGCGACGTCGCAG	ACGTTCCGGCCCGCCCGTT	T	C
K0011	ACCGAGTTCACCGGCAACTTC	CACCGAGTTCACCGGCAACTTT	GCAGGCACTGCGCGGCGAT	G	A
K0012	CGACGTCACGTGGCCGGA	CGACGTCACGTGGCCGGG	TACAGGAACGGCGGCCGAGTTA	T	C
K0013	CCGTCGTCGAGCACGGTG	GCCGTCGTCGAGCACGGTA	TGACACCACCGCCGTCCGTTT	C	T
K0014	GTGGCCTCAACTCCTTCTTCACT	GGCCTCAACTCCTTCTTCACC	CTCGAGGTAGTAGAAGAAGGAGTTCAT	T	C
K0015	GATTCTTTATCCGGTTAGTTCTGATTTG	CTTTATCCGGTTAGTTCTGATTTT	GCGTGGATCATCTGCAGCGCAA	C	A
K0016	CCTGTCCACGATAAAGATCTCGT	CCTGTCCACGATAAAGATCTCGA	TGACATCGGTTACGTGGATGACGAT	T	A
K0017	ACATGCTGCAGACACGGGGAT	CATGCTGCAGACACGGGGAC	AAGCAAAAATTGGCCGGTCATCAATACTA	T	C
K0018	AGGCCCTTCCGCTGGACG	AGGCCCTTCCGCTGGACC	ACGGTCTCCGCACAACGACGAA	G	C
K0019	TTCAAGTGGCCGGACCCTCA	CAAGTGGCCGGACCCTCC	CGATAACTCGGGCAGACGTCTATA	A	C
K0020[a]	**CAGCAGCAGCAGTCGGTGGT**	**CAGCAGCAGCAGTCGGTGGA**	**CAAATCTCAGCTGCCGCAGTATAGTA**	**T**	**A**
K0021	AAGTTTACGGCCGTTGCAGCGT	GTTTACGGCCGTTGCAGCGG	CCGTCGACGTCGTAGTTTGTTGATT	T	G
K0022	TTTACCAGGCGGAGTCCTTCG	GTTTTACCAGGCGGAGTCCTTCA	GCGTGCCTCGTGCCCTGGAA	G	A
K0023[a]	**GCTGCTGCAGAACCAGGG**	**CTGCTGCTGCAGAACCAGGC**	**AGGTGGCCCCGGCGCAGTA**	**G**	**C**

[a] Successful diagnostic marker.

Table 1.3 (Continued)

Primer ID	Allele X Primer	Allele Y Primer	Common Primer	Allele X	Allele Y
K0024	CTGGTAGAAGAGGCAGCACA	CTGGTAGAAGAGGCAGCACG	ACGTCGTCGGCATCGCCTTCTT	A	G
K0025	AGAAGACGTCCGATAACGCCTAT	GAAGACGTCCGATAACGCCTAC	CGGCGAGGGGCCGAAGCTA	A	G
K0026	AACCCTAGGTGGACGTCATCGA	AACCCTAGGTGGACGTCATCGT	TTTAATGACTAGGCCTACATGGTACGTAA	T	A
K0027[a]	**ATTCTGCAGCACCTGCAGCAC**	**CTGCAGCACCTGCAGCAG**	**CTGGATACTCATCCGGTGCGGTT**	**C**	**G**
K0028	AATGACGGGCCTATTCCTACGC	AATGACGGGCCTATTCCTACGG	ATTAACGACGTCATTGTCACGTTGAACAA	C	G
K0029	TCTGTACCACAAATTAGGATTCCC	ACTTCTGTACCACAAATTAGGATTCCT	TATGGGTGTGCCGGCTTCTTCTTT	C	T
K0030	GACTGCAGCAGGAGGAGACT	GACTGCAGCAGGAGGAGACC	CGGCTGCGTGGTCACCGGAA	T	C
K0031	CACCAACGACGACGTCTCCTTT	ACCAACGACGACGTCTCCTTC	GCTCATTTGTCGGCCCATGAAGAA	A	G
K0032	GGATCACCTGCAGCGCG	GCTGGATCACCTGCAGCGCA	CCGACGCCGGCCCCTTCAT	G	A
K0033[a]	**CCCGATGACCTGCAGCGACAA**	**CCGATGACCTGCAGCGACAC**	**GGCAACCAGGAGGTCGCCAA**	**A**	**C**
K0034	CCCTGGTGGGCGGCGG	CCCTGGTGGGCGGCGC	CGGCGGCACCACTGGCAGTT	G	C
K0035	GAAGCTGCTAAAGCTGTAGAGC	GAAGCTGCTAAAGCTGTAGAGG	TTCGGGGGCCGAACCTGAACAT	G	C
K0036	GCCAGCGGGCGGCTGC	CTGCCAGCGGGCGGCTGA	CAAGAGGCAGCAGCAGCAGCAA	G	T
K0037	CATCCCAAGGACCGGTTATGAG	CCATCCCAAGGACCGGTTATGAT	TTGCTCACACTGCAGCGATTTCGAT	C	A
K0038	TCCGGCGATATGCAGCG	GGCTTCCGGCGATATGCAGCT	CCCGCGCTTCGAGGCCGAA	C	A
K0039	CCTCCACCGTCACCTCCCA	CTCCACCGTCACCTCCCG	GACGGCCGTCTTCGACTGCTA	T	C
K0040	GGACCTTTGTTTCTGCAGGCTG	GGACCTTTGTTTCTGCAGGCTC	AATTGATGGCTACTATTAGTACACAGAGAA	G	C
K0041	GGCCGCAGCCTCTTCTACAAA	GGCCGCAGCCTCTTCTACAAC	GCCGTCGACGTCGGGAGGTA	A	C
K0042	CCGGAGTCGCAGCTGCGC	CCGGAGTCGCAGCTGCGT	ACCAGCTGCAGTGCGCGCTT	G	A
K0043	CACAGATTAGTGGCCAAACCGAG	CACAGATTAGTGGCCAAACCGAC	TGCAGAAACGTACTAAAGGTGAGACAAA	C	G
K0044	GCGCGCCGCTGCAGACAG	GCGCGCCGCTGCAGACAA	GTAGGTCGCTGGGTCCACGTAT	G	A
K0045	GGTAATAGGGCAGGGACGACT	GGTAATAGGGCAGGGACGACG	CGCGAATGACAGTAAGTACTTGCGTT	A	C
K0046	AGCGACCGGTTCTAGTGCTAGT	GCGACCGGTTCTAGTGCTAGG	TGTGCCTAGGGGCCACAGGAAA	A	C

[a] Successful diagnostic marker.

Results

Chromosome numbers and the nuclear DNA content

Among the 125 accessions examined in this study three ploidy levels were identified: diploid, triploid, and tetraploid. No higher ploidy levels were observed, though several studies reported pentaploid and hexaploid accessions (Taliaferro et al. 1997; Wu et al. 2006). Here, there were 41 diploids, 28 triploids, and 46 tetraploids. For nine accessions the ploidy levels could not be determined as the plants did not thrive in the hydroponic culture. Flow cytometry was attempted for 107 of the 125 accessions but the nuclear DNA content could not be measured for 18 accessions, because of insufficient plant material. The 2C nuclear DNA content ranged from 3.64 to 8.575 pg with an average of 5.4 pg. These values are substantially higher than any previous study (Eaton et al. 2004; Grossman et al. 2021; Johnson, Riordan, and Arumuganathan 1998). Hence, several accessions were selected at random to test for reproducibility of the method and the results were consistent. Based on these findings, somatic chromosome counts were deemed to be more reliable in determining ploidy levels among tested accessions. The chromosome counts and nuclear DNA values are listed in Table 1.2.

Morphology

The accessions in this study exhibited a wide range of morphological features. Based on visual evaluations most accessions were placed in one of

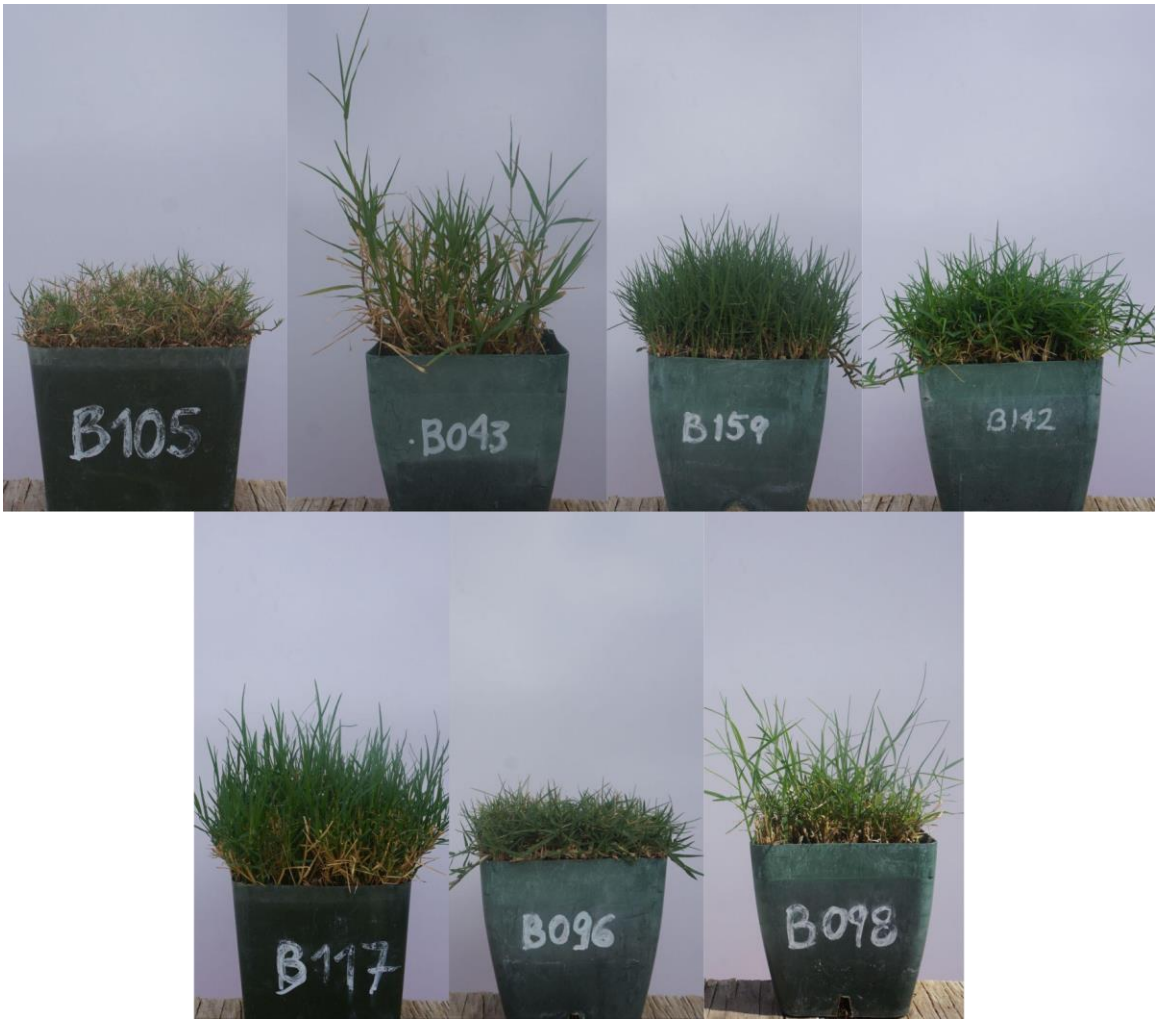

Figure 1.1 Examples of the seven major phenotypes observed among the accessions in this study. Plants are ordered corresponding to their morphological grouping and characteristics: group 1 (B105), group 2 (B043), group 3 (B159), group 4 (B142), group 5 (B117), group 6 (B096), and group 7 (B098).

seven groups (Figure 1.1). Some characteristics were shared among these groups, but members of each group had mostly similar phenotypes. Accessions in Group 1 had very fine leaves, slender red stolons, and an erect growth habit, which did not form a dense turf. Members of this group (B1, B4, B10, B13, B16, B30, B40, B50, B51, B52, B105, B126, B132, B147) were almost certainly

accessions of *C. transvaalensis*. This classification based on morphology aligned well with genetic groupings based on DNA polymorphism and ploidy levels, as well as previous descriptions of the species. Accessions placed in Group 2 had coarse, stiff leaves; thick stolons; and erect growth habit. It is unlikely that these accessions (B24, B43, B68c, B104, B140, B141) were the same species; they may be *C. aethiopicus*, *C. plectostachyus*, or *C. dactylon*. Accessions placed in Group 3 had fine leaves and short internodes that formed a low, dense turf. Members of this group (B31, B34, B45, B158, B159) were originally listed as *C. transvaalensis* and DNA polymorphism placed them in Group 1. However, chromosome counts have shown that they are either triploid or tetraploid, and exhibit features most similar to that of *C. dactylon*. Accessions in Group 4 had medium-sized leaves and short internodes forming a low, loose mat. Leaves were typically short, though they grew long when left unmowed. Members of this group (B8, B12, B14, B19, B20, B55, B61, B63, B65, B134, B135, B137, B142, B146, B156) grouped very closely based on the DNA polymorphism and geographic origins in India or Sri Lanka and are assumed to belong to *C. barberi*. Accessions in Group 5 had long, medium- to coarse-textured leaves and an erect growth habit, growing quite tall. Members of this group (B53, B60, B101, B106, B109, B116, B117, B129) are likely to belong to *C. dactylon*. Accessions in Group 6 had medium-textured leaves of short to medium length with short internodes. Accessions in Group 7 shared many characteristics of Group 6, but leaves tended to grow longer and rather loosely when left unmowed. Members of

Groups 6 (B2, B37, B48, B92, B95, B96, B97, B119, B125, B136) and 7 (B3, B9, B21, B27, B33, B35, B98, B102, B112, B124, B131) are likely to also belong to *C. dactylon*. Some accessions were quite unique, sharing characteristics with only one other accession, or none at all.

Genetic Marker Analysis

DArTseq generated a total of 376269 silicoDArT markers and 238167 SNPs, with the average reproducibility of 99.82% and 99.52%, respectively. After filtering, 17,959 silicoDArT and 6,920 SNP markers were retained for analyses and used in estimations of genetic distances among the accessions and various configurations of species assignment. After removing triploid and duplicate accessions, 86 accessions remained and were used in the remaining downstream analyses. The Mantel test for the silicoDArT- and SNP-based distance matrices showed a moderately strong positive correlation, with $r = .72$ ($P = .0001$). The UPGMA dendrograms derived from these distance matrices were similar within clusters but had minor differences between clusters. Unrooted dendrograms were also developed using the neighbor-joining (NJ) method. Little variation in actual clustering was observed between the UPGMA and NJ methods for both marker types, but differences in relatedness based on branch length were apparent given the nature of the two methods. UPGMA-based dendrograms were chosen over NJ-based to better visualize differences between groups/taxa.

Based on silicoDArT markers, genetic distances between species ranged from 0.141133 to 1.118544. The lowest genetic distance was between groups of accessions listed as *C. barberi* and *C. incompletus*. Non-significant genetic distance values were also observed. These values were all negative, with the lowest at -7.265071 between *C. aethiopicus* and *C. incompletus*. These *F*-statistic values are outside of their standard range of 0 to 1), but negative *F*-statistic values may result from unequal sample sizes between populations (Gerlach et al. 2010; Weir and Cockerham 1984). The genetic distance between accessions ranged from 0.012940 to 0.910668. The lowest genetic distance was between B4 and B28, both listed as *C. transvaalensis*). However, the DNA content data were dissimilar, highlighting differences between groupings based on silicoDArT or SNP markers. The largest calculated genetic distance was between B13 and B33 (both listed as *C. transvaalensis*). Morphologically, these two are different with B33 appearing to share some morphological characteristics, as well as genetic grouping, with *C. incompletus*. This is also consistent with the SNP-based UPGMA dendrogram (Figure 1.2).

Based on SNP markers, genetic distances between species ranged from 0.067536 to 0.742772. The lowest distance was between *C. barberi* and *C. incompletus*. This implies minimal or no genetic diversity between the two species and suggests that the few *C. incompletus* accessions present in the collection may actually be *C. barberi* accessions. This must not be taken as certain. *C. incompletus* was poorly represented in this study (as it is in the

source, USDA collection); a higher number of accessions could perhaps produce a better separation between the species.

The highest genetic distance was found to be between *C. plectostachyus* and *C. transvaalensis*. This is not surprising given contrasting morphology of the two species. A non-significant genetic distance value was observed between *C. dactylon* and *C. incompletus* (-0.003162). Again, a negative value in this case may well be a consequence of uneven representation of the two, and perhaps would be improved with more proportional sample sizes. The genetic distance between accessions ranged from 0.006011 to 0.563609. The smallest genetic distance was between B16 and B17 (both *C. transvaalensis* accessions collected by Dr. Jeff Krans), which might suggest that these two accessions may be collected from the same source. The largest genetic distance was between B13 and B139 (*C. transvaalensis* and *C. dactylon*, respectively). Visual morphological characters are nearly opposite between the two (e.g. fine vs coarse leaves, size, etc.).

The most probable numbers of clusters (*K*), as determined by Structure Harvester, differed between the silicoDArT and SNP data. For the SNP data, the best *K*-value was interpreted as $K = 3$, where $L(K)$ and the variance were -284235.6200 and 4232.4783, respectively. The $L(K)$ values of $K = 4$ and higher plateaued, while their respective variances increased (Figure 1.3).

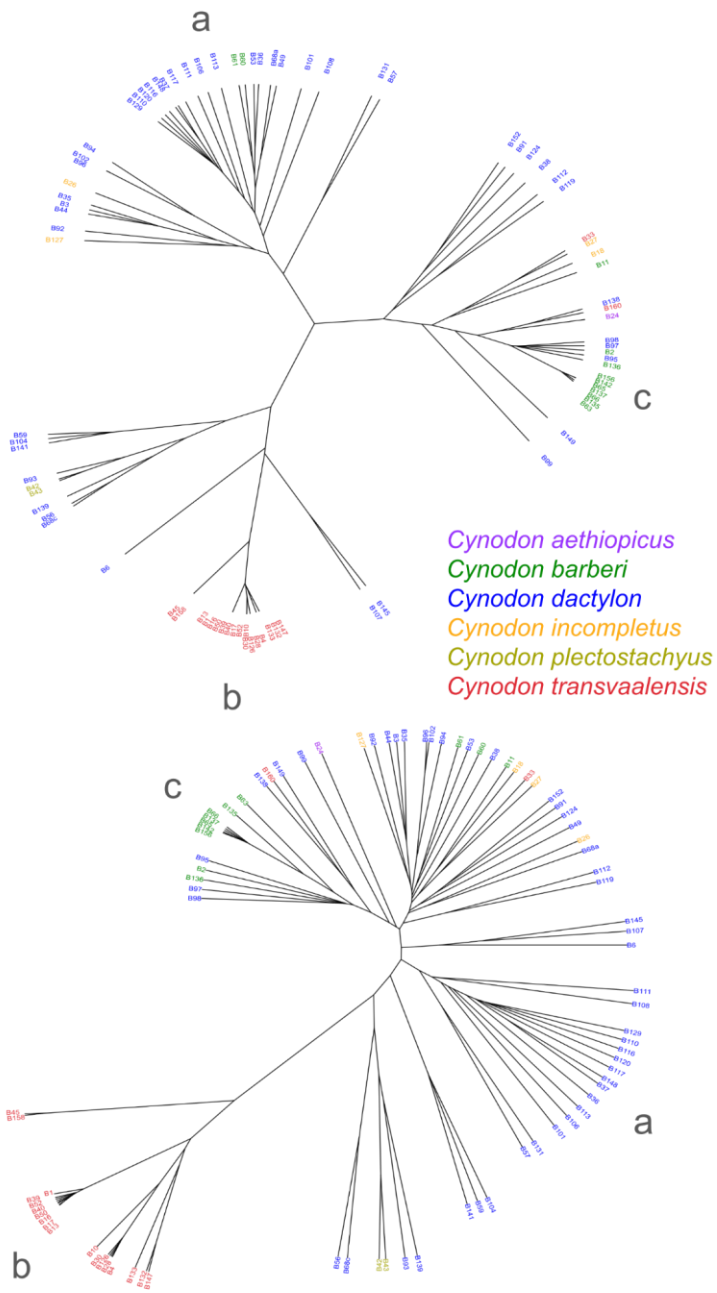

Figure 1.2 UPGMA dendrograms based on silicoDArT (top) or SNP (bottom) markers for 87 accessions from the UCR bermudagrass germplasm collection.
Group letters (a, b, c) correspond to the largest clusters within the dendrogram.

The Δ*K* values determined by Structure Harvester using the Evanno test peaked at *K* = 2 and sharply declined before *K* = 4, with a very small peak at *K* = 8. Visual comparisons between cluster graphs did not show much difference when K > 4, but can most likely be attributed to the low representation of the minor taxa (Figure 1.4). Results from Structure Harvester for the silicoDArT data were easier to interpret than those from the SNP data, where the best *K*-value was interpreted as *K* = 3. At *K* = 3, the *L*(*K*) and variance were -834628.5600 and 398.0007, respectively. *L*(*K*) values plateaued at *K* = 3, with minimal increase between *K* = 3 and *K* = 9. The Δ*K* values were close between *K* = 2 and *K* = 3, rapidly declining at *K* = 4.

Species-specific Markers

SNP markers specific for each species were identified. There were 221 such unique markers for *C. aethiopicus*, 31 for *C. barberi*, 1247 for *C. dactylon*, 29 for *C. incompletus*, 248 for *C. plectostachyus*, and 155 for *C. transvaalensis.* These markers were further filtered to remove those present in more than a single location in the genome. This was deemed necessary for the development of primers with easily scorable products. The numbers of single location markers, as determined by alignment to the ‘Tifway’ contig assembly were 18 for *C. aethiopicus*, 1 for *C. barberi*, 163 for *C. dactylon*, 1 for *C. incompletus*, 14 for *C. plectostachyus*, and 14 for *C. transvaalensis.* Although only one marker each was found for *C. barberi* and for *C. incompletus*, the idea

of relaxing the selection criteria to markers present in two or three locations was not implemented. From the 211 single-location markers, 46 candidates for species-specific markers were chosen at random to be used for primer development and KASP genotyping.

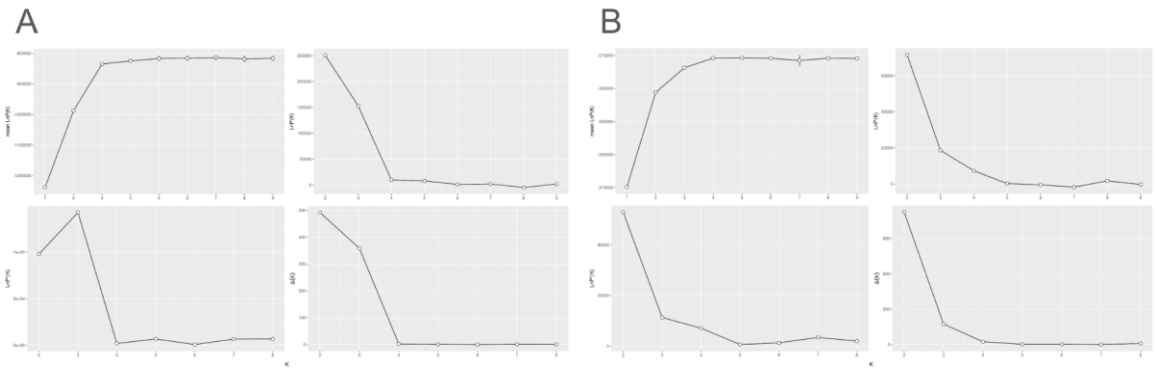

Figure 1.3 Graphical representation of the four steps of the Evanno method for detecting the true number of populations or clusters based on output from STRUCTURE for both silicoDArT (A) and SNP (B) data. For both A & B: (top left) mean likelihood *L*(*K*) over multiple runs for *K* = 1 to 9; (top right) rate of change for the likelihood function, *L*'(*K*); (bottom left) second order of rate change for the likelihood function, *L*''(*K*); (bottom right) Δ*K*- typical model choice criterion as discussed in Evanno et al. 2005.

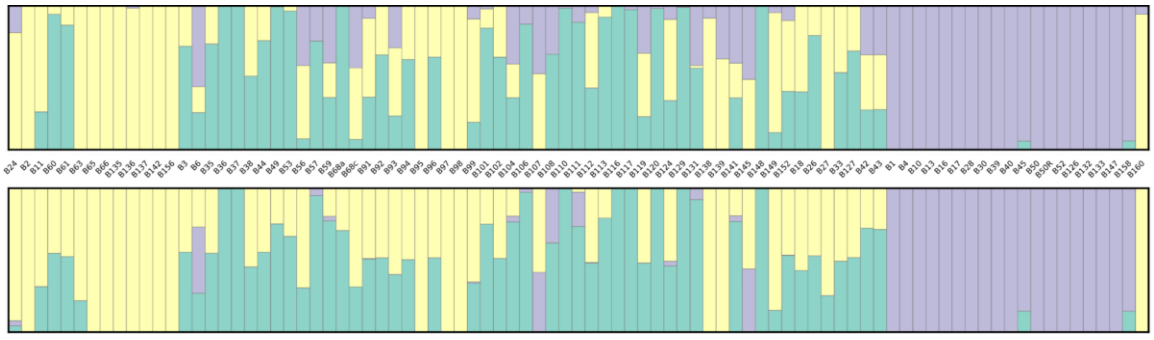

Figure 1.4 Cluster plots based on silicoDArT (top) and SNP (bottom) markers for 87 accessions at *K* = 3. Each color represents a different cluster in the population based on the output of STRUCTURE.

Of the 46 sequences sent to LGC Biosearch Technologies for primer development and KASP genotyping, four markers showed promise as potentially species-specific. More than half (24) of the marker sequences sent for KASP genotyping did not generate a consistent signal or did not amplify. Genotyping results for each marker were received from LGC Biosearch Technologies in the form of cluster plots (Figure 1.5). When accessions were genotyped using Marker K0020, all accessions were scored as T:T except for the two *C. plectostachyus*, which scored A:A. Similarly, all accessions scored G:G for Marker K0023 except for the two *C. plectostachyus* accessions, which scored C:G. Therefore, both Marker K0020 and K0023 may be considered as markers specific to the *C. plectostachyus* taxon.

Two other markers, K0027 and K0033, also showed promise. When genotyped with K0027, most but not all *C. barberi* accessions scored G:G (Figure 1.6). Two accessions outside of the *C. barberi* taxon, B20 (*C. transvaalensis*) and B139 (*C. dactylon*), also scored G:G for this marker. B20 was identified as a triploid but grouped very closely with *C. barberi* accessions prior to the removal of triploid accessions from the final study group. B139, however, is genetically distant from the cluster of *C. barberi* and groups closer with *Cynodon* accessions with much wider leaves, such as *C. plectostachyus* and some broad-leafed *C. dactylon* accessions. Accessions listed as *C. barberi* that did not score G:G for K0027 included B2, B9, B21, B61, and B136. Although B2 and B136 are relatively close with the *C. barberi* cluster, the two accessions group close with *C.*

dactylon accessions originating from India. B61 grouped closely with another cluster of *C. dactylon* accessions collected from India, though these accessions were more genetically distant from the *C. barberi* cluster. B9 and B21 were identified as triploids, though they grouped similarly with B2 and B136 prior to the filtering of triploids from downstream analyses. These results suggest that K0027 is indeed *C.barberi*-specific and that some additional revisions are necessary for this group of accessions.

When genotyped with Marker K0033, most *C. transvaalensis* accessions, but not all, scored A:A. No accessions from other species, however, scored A:A such as in the case of B20 and B139 for K0027. Accessions listed as *C. transvaalensis* that did not score A:A for 3662-0120 included B20, B31, B33, B34, B45, B133, B158, and B159. B20, B31, B34, and B159 turned out to be triploids. Prior to their removal from downstream analyses, B20 grouped with accessions within the *C. barberi* cluster, while B31, B34, and B159 grouped with a small cluster of *C. dactylon* accessions close to the *C. transvaalensis* cluster. After revisiting B45 and B158, we identified two mislabeled pots from which the ploidy and morphological data were collected. Removal of these accessions and subsequent revaluation led us to confirm these accessions as *C. dactylon*. Similar to K0027, these results suggest that K0033 may be a *C. transvaalensis*-specific marker and that additional revisions may be required for accessions scored otherwise. However, one interesting accession in this list is B133. It is

listed as a *C. transvaalensis* accession, is diploid, and based on genetic and morphological data it groups with the *C. transvaalensis* cluster, yet it does not appear to carry the same allele as the others, to score A:A in this analysis.

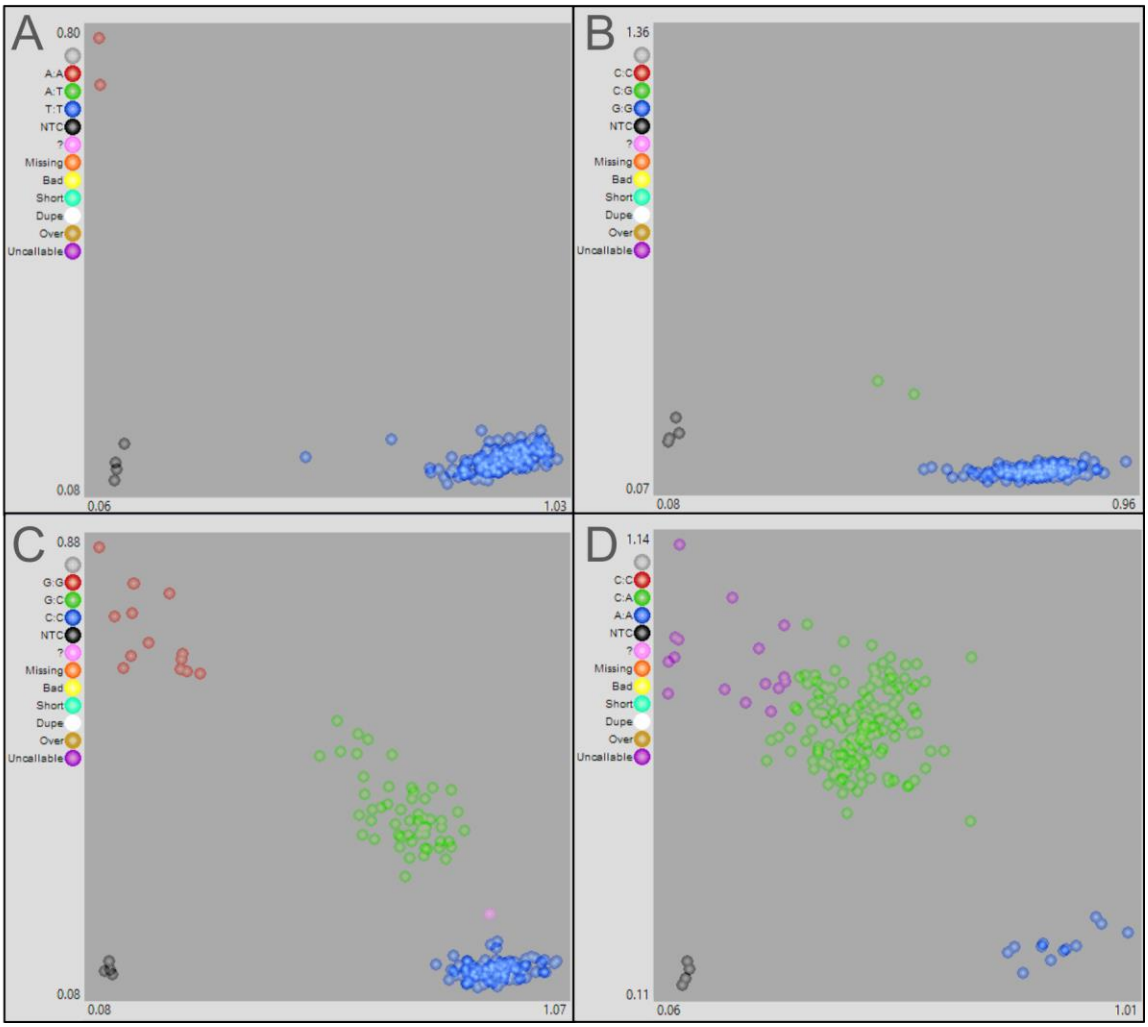

Figure 1.5 Scatter plot outputs from KASP genotyping. Because two 96-well plates were used for genotyping, plots for Plate 2 were overlaid on top of Plate 1 at 50% opacity. For example, in (A) one *C. plectostachyus* accession was genotyped on Plate 1 while the other was on Plate 2, thus prompting the need for overlays. Axes and fluorescence intensities were the same between both plates. (A) K0020, marker specific to *C. plectostachyus* when scored A:A (red). (B) K0023, marker specific to *C. plectostachyus* when scored C:G (green). (C) K0027, marker specific to *C. barberi* when scored G:G (red). (D) K0033, marker specific to *C. transvaalensis* when scored A:A (blue).

This may well be a mutation at this locus. Another possibility might be that the species-specific markers show different levels of efficacy. Whatever the reasoning for B133's off-type with K0033, we believe that these findings show promise as a step towards identifying effective markers for identifying other *Cynodon* taxa and it needs to be stressed again that this study was intended from the start as an exploration and a proof-of-concept of the approach.

Species reclassification

A change in species listing was recommended for 30 accessions. These changes were based on the following: 1) ploidy levels that did not match the literature-listed values, 2) some distinct morphological traits typical of another species, and 3) genetic relatedness. Accessions B3, B35, B36, B37, B38, B131, B158, B160, and B162 were obtained from sources (Table 1.1) as *C. transvaalensis*, but based on analyses performed here, are proposed to be reclassified as *C. dactylon*. They are tetraploid, sharing some morphological characteristics, and genetically group with *C. dactylon* accessions. Similarly, accessions B28, B50, B52, B105, B126, B133, and B147 obtained from sources as *C. dactylon* must be reclassified as *C. transvaalensis*. They are diploid, sharing morphological characteristics and genetic grouping with *C. transvaalensis* accessions. In some cases, ploidy level could not be used as a distinguishing factor. For example, B156, obtained here as *C. radiatus* should probably be classified as a *C. barberi* based on morphological characteristics and

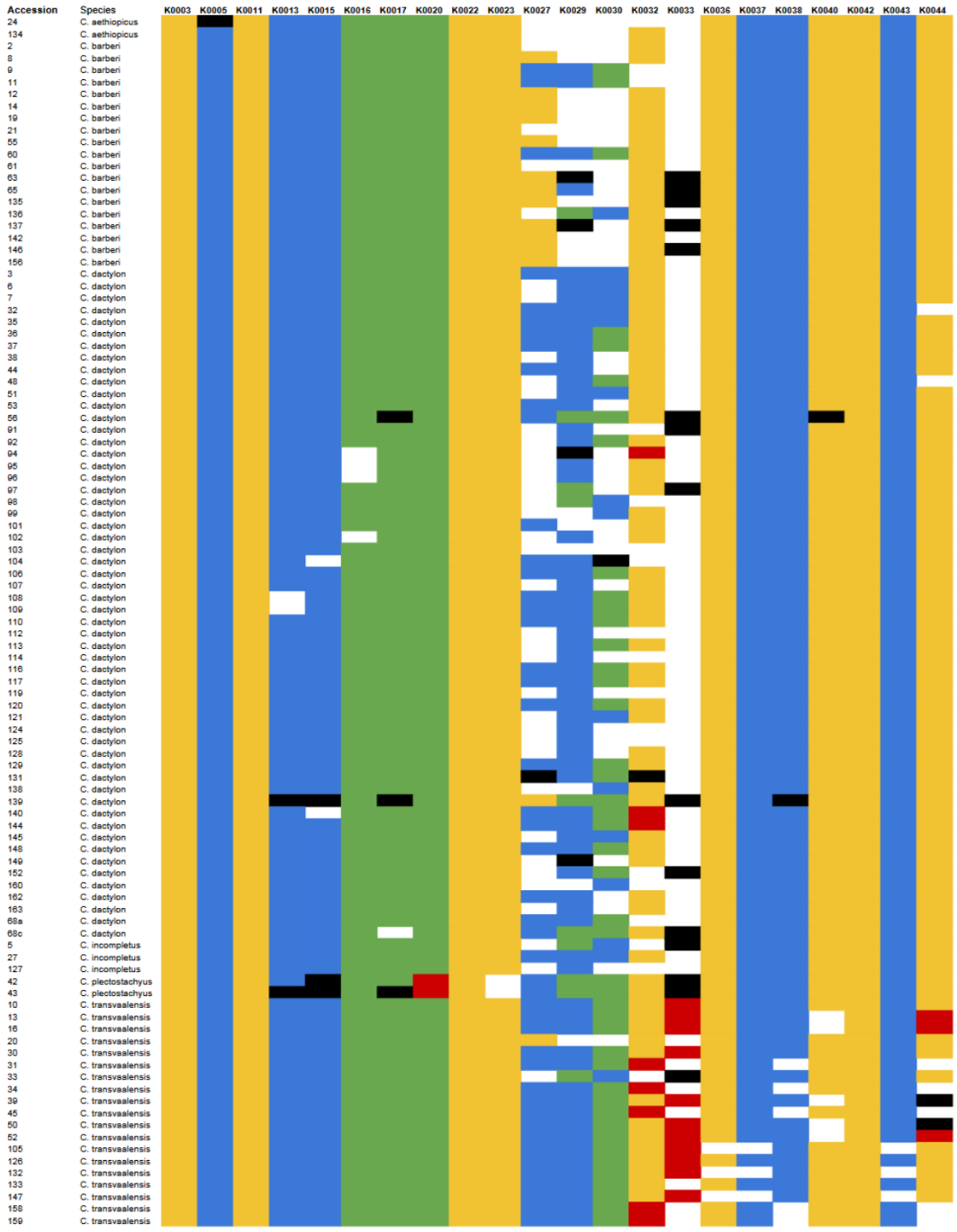

Figure 1.6 Visualization of haplotype scoring for 22 of 46 KASP markers. KASP markers that did not amplify for any accession were removed. When all accessions (rows) of a particular species shared the same score for a single KASP marker (columns), the KASP marker was considered to be species-specific. Colors represent the following scores: yellow = G:G, blue = C:C, green = T:T, red = A:A, white = heterozygous, black = missing or no call.

genetic grouping. Both *C. radiatus* and *C. barberi* are diploid taxa, but B156 clustered very closely with *C. barberi* accessions B65, B66, B137, and B142 based on genetic markers. All species recommendations are listed in Table 1.2.

Discussion

Ensuring the accurate identification of species within a germplasm collection is critical, and can limit its utility to researchers. Challenges may arise in correctly identifying different species for both germplasm repositories and breeding programs, especially in situations where genetic resources are missing and collection managers must rely on visual assessments of morphological features (Mason et al. 2015).

The author will not be the first to state that species classification in the genus *Cynodon* is in serious disarray. Not only do some species lack clearly discriminating morphological features, but the presence of such a high proportion of triploids shows that interspecific hybridization is frequent. Whether this is an issue of collection maintenance, or a natural phenomenon in natural stands, is an open question. Triploids originate from mating of tetraploids with diploids. If this is so frequent, it cannot be assumed that interspecific hybridization among diploids and tetraploids does not occur as frequently, or even more so. Perhaps the only clarification could be made by re-collecting the specimen from their natural environments, followed by immediate analyses by various means, starting from chromosome counts and DNA diversity studies. An additional complication here

is that the genomic assembly available for this study was produced from a triploid interspecific hybrid. It is unknown whether this approach (using a triploid genome to develop species-specific markers) can yield species-specific markers that can be traced to their respective subgenomes.

Genetic diversity studies of bermudagrass germplasm collections have featured common bermudagrass (*C. dactylon*) and/or African bermudagrass (*C. transvaalensis*) accessions, with few studies including the other *Cynodon* taxa. Although genetic markers have been utilized to distinguish between bermudagrass cultivars, these studies generally use small sample sizes and typically do not include experimental lines (Harris-Shultz, Schwartz, and Brady 2011; Wang et al. 2010; Yang et al. 2018). To the best of our knowledge, Pudzianowska and Baird (2021) were the first to identify (but not develop) a number of species-specific markers for members of the *Cynodon* genus, though such diagnostic markers have been reported in a few plant genera such as *Eucalyptus*, *Pinus*, *Citrus*, *Brassica,* and *Oryza* (Balasaravanan et al. 2006; Cullingham et al. 2013; Curk et al. 2015; Mason et al. 2015; Ndjiondjop et al. 2018). Based on this study, 30 accessions within the bermudagrass germplasm collection at UCR were reclassified (given what appears to be their correct species assignments). This highlights the importance of a multipronged approach needed for proper species identification. In addition, we explore the potential for the development of species-specific genetic markers in the *Cynodon* genus and demonstrated that indeed this approach can be successful in the development of

diagnostic markers. Here, the handful of markers discriminated accessions of three members of the genus: *C. barberi*, *C. plectostachyus*, and *C. transvaalensis*.

As stated above, a large number of accessions in the UCR collection, all obtained from other established collections, either directly or indirectly, are triploid. It is impossible at this stage to determine their exact origin. Perhaps such interspecific hybridization occurs among natural stands, were collected and classified based on morphological similarities to pure species or occurred in the collections themselves. Since these are living collections (as opposed to seed storage), on small plots on limited area, chances for hybridization appear greater than in the natural stands. Hybrid seed may drop to the ground and germinate, producing seedlings capable of outcompeting the original female parent. Whether such hybridization occurred at the repository level (USDA) or at UCR (or both) is an open question but some early identified questionable accessions (those that did not group properly on first dendrograms produced at UCR), were re-ordered from USDA, and the duplicates produced the same exact groupings (Pudzianowska and Baird 2021). The *Cynodon* taxon, *C.* × *magennisii*, is suspected to be a naturally occurring hybrid between a tetraploid *C. dactylon* and a diploid *C. transvaalensis*, producing a sterile triploid, and is endemic to South Africa where the two species occur sympatrically. Literature proposing nine *Cynodon* species included *C.* × *magennisii*- as a species in its own right but this is subject of dispute. This species has an extremely narrow endemic region and

is sterile by nature, raising the question whether it is a valid species. Naturally occurring triploids have been reported in wild populations of other plant species, with some being designated as a named species (*Cynodon* × *magennisii*) rather than as only a product of hybridization (*C. dactylon* × *C. transvaalensis*) (Grant 1981; Vallejo-Marín et al. 2016). Based on the International Code of Nomenclature for algae, fungi, and plants (Shenzen Code), hybrids derived from recognized taxa where at least one parental taxon is known can be designated with the multiplication sign "×" or referred to as "nothotaxa" (Turland et al. 2018). However, they also note "taxa that are believed to be of hybrid origin need not be designated as nothotaxa", leaving the nomenclature to the subjective opinion of taxonomists (Turland et al. 2018). Indeed, confusion has risen among taxonomists regarding the proper usage of this nomenclature (Parkinson 1985; Wieczorek 2023).

In breeding programs, triploid accessions have been intentionally generated for their improved quality and features (e.g. heterosis, sterility, etc.), and as such are typically a product of a breeding program, rather than material used in one. Overall, about 22% of the 125 collection accessions were identified as triploids in this study, greatly reducing the range of variation and the genetic diversity assumed by Pudzianowska and Baird (2021) for this collection. Most of these were previously listed as either *C. dactylon* or *C. barberi* accessions. With the large amount of genetic diversity found within the *C. dactylon* taxon and the potential for *C. dactylon* to hybridize with three other *Cynodon* taxa, difficulties in

properly classifying bermudagrass accessions are almost expected and highlight the need for genetic markers that would enable genotyping for species identification. While successful hybridizations between triploids and other ploidy levels have been reported in other plants (Husband 2004; Kovalsky et al. 2018; Li et al. 2022), generating the large number of crosses needed to overcome extremely low fertilization rates can be quite costly to a breeding program and ultimately do not justify the retention of triploid material. Another 23% of the 125 collection accessions are believed to be misclassified- a high proportion, but not unheard of in other studies (Buso, Rangel, and Ferreira 2001; Girma et al. 2012; Mason et al. 2015; Orjuela et al. 2014).

Similar to Pudzianowska and Baird (2021), clustering from UPGMA and population structure analyses seemed to differentiate accessions into three large clusters with possible subclusters. This clustering was more obvious in the silicoDArT UPGMA (Figure 1.2). The true value of *K* can be identified using the maximal value of the mean posterior probabilities ($L(K)$) (Ciofi et al. 2002; Hampton et al. 2004; Vernesi et al. 2003; Zeisset and Beebee 2001) or the value at which $L(K)$ begins to plateau (Evanno, Regnaut, and Goudet 2005; Pritchard et al. 2000). Although the most probable value of *K* was determined to be $K = 2$ (based on the Evanno test for ΔK) (Figure 1.3) we believe that $K = 3$ seems more intuitive based on biological characters and visual assessment of cluster and UPGMA graphs. Though the popular “Evanno test” determines the best value of *K* as the modal value for the distribution of ΔK (where the height of the value can

be used as an indicator of strength), it should not be used exclusively in determining the best value of *K* (Evanno et al. 2005; Janes et al. 2017). In many cases, *K* = 2 represents the uppermost level of population structuring and can be influenced by sampling within the population (Evanno et al. 2005; Janes et al. 2017). This can also be indicative of sublevels within the population structure, which were more apparent in the silicoDArT UPGMA dendrogram. Additionally, STRUCTURE has been known to bias lower *K*-value estimates of the number of subpopulations when sample sizes are uneven between subpopulations, as is the case in our list of accessions with minimal representation from the minor *Cynodon* taxa (Puechmaille 2016). Should the opportunity arise to perform a similar study with larger numbers of accessions from the minor *Cynodon* taxa, greater resolution between the subpopulations and species groupings would almost certainly happen. This is not an easy proposition and these minor taxa are poorly represented in established collections.

Morphological groupings appeared to match well the observed genetic groupings from both the silicoDArT and SNP dendrograms. UPGMA based on the dominant silicoDArT markers appeared to better distinguish groups based on similar morphologies than SNP markers. The grouping of accessions based on similar phenotypes was done with the intent to identify misclassified accessions, not to assign a species to multiple accessions based on broad characters. Taxonomic classifications are much more nuanced, though they can often lead to confusion and difficulty in species identification (Hurcombe 1948; de Wet and

Harlan 1970), and individual attention was given to each accession listed. *Cynodon* taxa, especially *C. dactylon*, exhibit a very wide phenotypic variation and consequently some of the taxa can be further classified into varieties. No attempts were made here to distinguish varieties because the main goal was to clarify species classifications for breeding program and to explore the possibility of developing diagnostic genetic markers. Further reduction of the *Cynodon* taxa into their respective varieties would only lower representation, making it nearly impossible to identify species-specific markers.

Although the 2C nuclear DNA content was not used to validate the ploidy levels of our accessions, such values could be used to clarify inconsistencies among accession groupings. Accessions B28 and B133 both grouped as *C. transvaalensis*, yet their 2C values (4.15 and 3.95 pg, respectively) do not fall within the range of other closely related *C. transvaalensis* accessions (7.30 to 8.58 pg). The author has no clear explanation for such a difference but some contamination or mislabeling cannot be entirely excluded, given the number of accessions under investigation, and the history of these accessions. The 2C values of all other accessions were relatively consistent within similarly grouped accessions. These values also seemed consistent with morphological groupings. Accessions that were grouped together based on genetic markers usually shared a similar geographic origin as has been previously discussed (Pudzianowska and Baird 2021).

The cost of generating genetic markers continues to go down as sequencing methods become more efficient. The implementation of KASP genotyping for the development of diagnostic SNPs has been discussed previously by Ndjiondjop et al. (2018). In their study, 332 diagnostic SNPs were identified across the *Oryza* genome, and 36 of these markers were recommended for genotyping purposes (Ndjiondjop et al. 2018). The basis for their marker selection criteria are described by Semagn et al. (2012): developing effective diagnostic markers requires 1) a small number of markers to be cost-effective; 2) markers with the ability to differentiate between homozygous and heterozygous genotypes; 3) markers with minor allele frequency and PIC values of at least 0.20 and 0.25, respectively; and 4) uniform distribution of markers across chromosomes (Semagn et al. 2012). In this study, the presence of tetraploids eliminated the chance to develop markers differentiating between homozygous and heterozygous genotypes. Although the reference assembly used in this study belongs to a bermudagrass hybrid (*C. dactylon* × *C. transvaalensis*) and was divided into its subgenomes, it is insufficient to determine which subgenome each SNP marker belongs to. By definition, species-specific markers ought to be genome-specific. Additionally, there are no genomic resources for any of the minor *Cynodon* taxa (*C. aethiopicus, C. barberi, C. incompletus, C. nlemfluensis, C. plectostachyus, C. radiatus*), and studies detailing their evolution are scarce. The low representation of the minor taxa plays a larger role in diagnostic marker discovery than initially expected. When

using nine fewer accessions (78 total), two of the four working diagnostic markers were not informative, as they turned out not to be species-specific. It would seem that the inclusion of as many accessions as possible would aid in the discovery of species-specific markers, further highlighting the need for greater representation of minor *Cynodon* taxa.

As of April 2024, 203 *Cynodon* accessions are available through the USDA-ARS NPGS. Of those listed, there are 2 accessions of *C.* × *magenissii*, 1 accession of *C. aethiopicus*, 8 accessions of *C. barberi*, 146 accessions of *C. dactylon*, 3 accessions of *C. incompletus*, 2 accessions of *C. nlemfuensis*, 10 accessions of *C. plectostachyus*, 7 accessions of *C. radiatus*, and 13 accessions of *C. transvaalensis*, as well as an additional 8 *Cynodon* accessions where the species was unknown. This does not include historic accessions for which only information remains; there are no physical plants. The availability of accessions belonging to minor *Cynodon* taxa is sparse, though it is unknown how many of these accessions can be found in germplasm collections at other universities, private collections, etc. Although much genetic diversity exists within the metropolitan *C. dactylon* species, the exclusion of the minor *Cynodon* taxa from research studies and breeding programs may be a lost opportunity to develop novel hybrids with improved features or stress tolerance. Preservation of these taxa is important, and the number of taxonomists with formal training in identifying these species is decreasing. Efforts should be made to at least generate intraspecific hybrids.

Conclusion

The results illustrated challenges in discriminating between different species of *Cynodon* and the need for more objective methods for species classifications. A large proportion of accessions studied here were identified as triploids, prompting their removal from future breeding efforts and posing a question to how triploids should be classified taxonomically. Various genomic tools were introduced and tested which may assist breeders' discrimination among minor *Cynodon* taxa and in making rational decisions about parent selecting in their breeding programs. Results from this study reiterate once again the importance of sample size when developing diagnostic genetic markers, and identification of four species specific DNA markers in the very first exploratory attempt is promising. Future efforts should be made to identify and expand the number of accessions from minor *Cynodon* taxa, if they can be found, and species-specific diagnostic markers revisited when such studies can be done with greater confidence enhanced by increased/equal species representation.

References

Assefa, S., C. M. Taliaferro, M. P. Anderson, B. G. de los Reyes, and R. M. Edwards. 1999. “Diversity among Cynodon Accessions and Taxa Based on DNA Amplification Fingerprinting.” Genome 42(3):465–74. doi: 10.1139/g98-152.

Assefa, S., Taliaferro, C. M., Anderson, M. P., de los Reyes, B. G., & Edwards, R. M. (1999). Diversity among Cynodon accessions and taxa based on DNA amplification fingerprinting. Genome, 42(3), 465–474. https://doi.org/10.1139/g98-152

Balasaravanan, T., Chezhian, P., Kamalakannan, R., Yasodha, R., Varghese, M., Gurumurthi, K., & Ghosh, M. (2006). Identification of Species-Diagnostic ISSR Markers for Six Eucalyptus Species. Silvae Genetica, 55(1–6), 119–122. https://doi.org/10.1515/sg-2006-0017

Burton, G. W. (1966). Tifway (Tifton 419) bermudagrass (Reg. No. 7).

Buso, G. S. C., Rangel, P. H. N., & Ferreira, M. E. (2001). Analysis of random and specific sequences of nuclear and cytoplasmic DNA in diploid and tetraploid American wild rice species (Oryza spp.). Genome, 44(3), 476–494. https://doi.org/10.1139/gen-44-3-476

Chiavegatto, R. B., Paula, C. M. P., Souza Sobrinho, F., Benites, F. R. G., & Techio, V. H. (2016). Karyotype asymmetry in Cynodon Rich. (Poaceae) accessions. Genetics and Molecular Research, 15(4). https://doi.org/10.4238/gmr15049152

Ciofi, C., Milinkovitch, M. C., Gibbs, J. P., Caccone, A., & Powell, J. R. (2002). Microsatellite analysis of genetic divergence among populations of giant Galápagos tortoises. Molecular Ecology, 11(11), 2265–2283. https://doi.org/10.1046/j.1365-294X.2002.01617.x

Cullingham, C. I., Cooke, J. E. K., Dang, S., & Coltman, D. W. (2013). A species-diagnostic SNP panel for discriminating lodgepole pine, jack pine, and their interspecific hybrids. Tree Genetics & Genomes, 9(4), 1119–1127. https://doi.org/10.1007/s11295-013-0608-x

Curk, F., Ancillo, G., Ollitrault, F., Perrier, X., Jacquemoud-Collet, J.-P., Garcia-Lor, A., Navarro, L., & Ollitrault, P. (2015). Nuclear Species-Diagnostic SNP Markers Mined from 454 Amplicon Sequencing Reveal Admixture Genomic Structure of Modern Citrus Varieties. PLOS ONE, 10(5), e0125628. https://doi.org/10.1371/journal.pone.0125628

De Candolle, A. P., & de Candolle, A. (1844). Théorie élémentaire de la botanique, ou, Exposition des principes de la classification naturelle et de l'art de décrire et d'étudier les végétaux. Roret.

de Wet, J. M. J., & Harlan, J. R. (1970). Biosystematics of Cynodon L. C. Rich. (Gramineae). TAXON, 19(4), 565–569. https://doi.org/10.2307/1218950

Dray, S., & Dufour, A.-B. (2007). The ade4 Package: Implementing the Duality Diagram for Ecologists. Journal of Statistical Software, 22, 1–20. https://doi.org/10.18637/jss.v022.i04

Earl, D. A., & vonHoldt, B. M. (2012). STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources, 4(2), 359–361. https://doi.org/10.1007/s12686-011-9548-7

Eaton, T. D., Curley, J., Williamson, R. C., & Jung, G. (2004). Determination of the Level of Variation in Polyploidy among Kentucky Bluegrass Cultivars by Means of Flow Cytometry. Crop Science, 44(6), 2168–2174. https://doi.org/10.2135/cropsci2004.2168

Evanno, G., Regnaut, S., & Goudet, J. (2005). Detecting the number of clusters of individuals using the software structure: A simulation study. Molecular Ecology, 14(8), 2611–2620. https://doi.org/10.1111/j.1365-294X.2005.02553.x

Gerlach, G., Jueterbock, A., Kraemer, P., Deppermann, J., & Harmand, P. (2010). Calculations of population differentiation based on GST and D: Forget GST but not all of statistics! Molecular Ecology, 19(18), 3845–3852. https://doi.org/10.1111/j.1365-294X.2010.04784.x

Girma, G., Korie, S., Dumet, D., & Franco, J. (2012). Improvement of accession distinctiveness as an added value to the global worth of the yam (Dioscorea spp) genebank.

Grant, V. (1981). 34. Natural Triploids. In Plant Speciation (pp. 473–478). Columbia University Press. https://doi.org/doi:10.7312/gran92318-035

Grossman, A. Y., Andrade, M. H. M. L., Chaves, A. L. A., Mendes Ferreira, M. T., Techio, V. H., Lopez, Y., Begcy, K., Kenworthy, K. E., & Rios, E. F. (2021). Ploidy Level and Genetic Parameters for Phenotypic Traits in Bermudagrass (Cynodon spp.) Germplasm. Agronomy, 11(5), 912. https://doi.org/10.3390/agronomy11050912

Gruber, B., Unmack, P. J., Berry, O. F., & Georges, A. (2018). dartr: An r package to facilitate analysis of SNP data generated from reduced representation genome sequencing. Molecular Ecology Resources, 18(3), 691–699. https://doi.org/10.1111/1755-0998.12745

Haider, N. (2018). A brief review on plant taxonomy and its components. The Journal of Plant Science Research, 34(2), 277–292.

Hampton, J. O., Spencer, P. B. S., Alpers, D. L., Twigg, L. E., Woolnough, A. P., Doust, J., Higgs, T., & Pluske, J. (2004). Molecular techniques, wildlife management and the importance of genetic population structure and dispersal: A case study with feral pigs. Journal of Applied Ecology, 41(4), 735–743. https://doi.org/10.1111/j.0021-8901.2004.00936.x

Hanna, W., Raymer, P., & Schwartz, B. (2013). Warm-Season Grasses: Biology and Breeding. In Turfgrass: Biology, Use, and Management (pp. 543–590). https://doi.org/10.2134/agronmonogr56.c16

Harlan, J. R., de Wet, J. M. J., Huffine, W. W., & Deakin, J. R. (1970). A guide to the species of Cynodon (Gramineae). Bulletin of the Oklahoma Agricultural Experiment Station, B-673.

Harris-Shultz, K. R., Schwartz, B. M., & Brady, J. A. (2011). Identification of Simple Sequence Repeat Markers that Differentiate Bermudagrass Cultivars Derived from 'Tifgreen.' Journal of the American Society for Horticultural Science, 136(3), 211–218. https://doi.org/10.21273/JASHS.136.3.211

He, C., Holme, J., & Anthony, J. (2014). SNP Genotyping: The KASP Assay. In D. Fleury & R. Whitford (Eds.), Crop Breeding: Methods and Protocols (pp. 75–86). Springer. https://doi.org/10.1007/978-1-4939-0446-4_7

Hennig, W. (1999). Phylogenetic systematics. University of Illinois Press.

Heywood, V. H., & Watson, R. T. (1995). Global biodiversity assessment (Vol. 1140). Cambridge university press Cambridge.

Hilu, K. W. (2007). A Century of Progress in Grass Systematics. Kew Bulletin, 62(3), 355–373.

Hulse-Kemp, A. (2022). *'Tifway' reference contig assembly* [Unpublished data]. USDA-Agricultural Research Service.

Hurcombe, R. E. (1948). A cytological and morphological study of cultivated Cynodon species. In Better turf through research (pp. 36–47).

Husband, B. C. (2004). The role of triploid hybrids in the evolutionary dynamics of mixed-ploidy populations. Biological Journal of the Linnean Society, 82(4), 537–546. https://doi.org/10.1111/j.1095-8312.2004.00339.x

Janes, J. K., Miller, J. M., Dupuis, J. R., Malenfant, R. M., Gorrell, J. C., Cullingham, C. I., & Andrew, R. L. (2017). The K = 2 conundrum. Molecular Ecology, 26(14), 3594–3602. https://doi.org/10.1111/mec.14187

Jewell, M. C., Zhou, Yi., Loch, D. S., Godwin, I. D., & Lambrides, C. J. (2012). Maximizing Genetic, Morphological, and Geographic Diversity in a Core Collection of Australian Bermudagrass. Crop Science, 52(2), 879–889. https://doi.org/10.2135/cropsci2011.09.0497

Johnson, P. G., Riordan, T. P., & Arumuganathan, K. (1998). Ploidy Level Determinations in Buffalograss Clones and Populations. Crop Science, 38(2), cropsci1998.0011183X003800020034x. https://doi.org/10.2135/cropsci1998.0011183X003800020034x

Kilian, A., Wenzl, P., Huttner, E., Carling, J., Xia, L., Blois, H., Caig, V., Heller-Uszynska, K., Jaccoud, D., Hopper, C., Aschenbrenner-Kilian, M., Evers, M., Peng, K., Cayla, C., Hok, P., & Uszynski, G. (2012). Diversity Arrays Technology: A Generic Genome Profiling Technology on Open Platforms. In F. Pompanon & A. Bonin (Eds.), Data Production and Analysis in Population Genomics: Methods and Protocols (pp. 67–89). Humana Press. https://doi.org/10.1007/978-1-61779-870-2_5

Kovalsky, I. E., Roggero Luque, J. M., Elías, G., Fernández, S. A., & Solís Neffa, V. G. (2018). The role of triploids in the origin and evolution of polyploids of Turnera sidoides complex (Passifloraceae, Turneroideae). Journal of Plant Research, 131(1), 77–89. https://doi.org/10.1007/s10265-017-0974-9

Kumpatla, S. P., Buyyarapu, R., Abdurakhmonov, I. Y., & Mammadov, J. A. (2012). Genomics-Assisted Plant Breeding in the 21st Century: Technological Advances and Progress. In Plant Breeding. IntechOpen. https://doi.org/10.5772/37458

Li, M.-M., Su, Q.-L., Zu, J.-R., Xie, L., Wei, Q., Guo, H.-R., Chen, J., Zeng, R.-Z., & Zhang, Z.-S. (2022). Triploid cultivars of Cymbidium act as a bridge in the formation of polyploid plants. Frontiers in Plant Science, 13. https://doi.org/10.3389/fpls.2022.1029915

Madden, T. (2013). The BLAST sequence analysis tool. The NCBI Handbook, 2, 425–436.

Mason, A. S., Zhang, J., Tollenaere, R., Vasquez Teuber, P., Dalton-Morgan, J., Hu, L., Yan, G., Edwards, D., Redden, R., & Batley, J. (2015). High-throughput genotyping for species identification and diversity assessment in germplasm collections. Molecular Ecology Resources, 15(5), 1091–1101. https://doi.org/10.1111/1755-0998.12379

Ndjiondjop, M. N., Semagn, K., Zhang, J., Gouda, A. C., Kpeki, S. B., Goungoulou, A., Wambugu, P., Dramé, K. N., Bimpong, I. K., & Zhao, D. (2018). Development of species diagnostic SNP markers for quality control genotyping in four rice (Oryza L.) species. Molecular Breeding, 38(11), 131. https://doi.org/10.1007/s11032-018-0885-z

Orjuela, J., Sabot, F., Chéron, S., Vigouroux, Y., Adam, H., Chrestin, H., Sanni, K., Lorieux, M., & Ghesquière, A. (2014). An extensive analysis of the African rice genetic diversity through a global genotyping. Theoretical and Applied Genetics, 127(10), 2211–2223. https://doi.org/10.1007/s00122-014-2374-z

Parkinson, P. G. (1985). (51)-(56) Problems with the Nomenclature of Nothotaxa in the Sydney Code with Six Proposals to Amend. Taxon, 34(2), 334–337. https://doi.org/10.2307/1221814

Peterson, P. M., Romaschenko, K., & Johnson, G. (2010). A classification of the Chloridoideae (Poaceae) based on multi-gene phylogenetic trees. Molecular Phylogenetics and Evolution, 55(2), 580–598. https://doi.org/10.1016/j.ympev.2010.01.018

Pina-Martins, F., Silva, D. N., Fino, J., & Paulo, O. S. (2017). Structure_threader: An improved method for automation and parallelization of programs structure, fastStructure and MavericK on multicore CPU systems. Molecular Ecology Resources, 17(6), e268–e274. https://doi.org/10.1111/1755-0998.12702

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of Population Structure Using Multilocus Genotype Data. Genetics, 155(2), 945–959. https://doi.org/10.1093/genetics/155.2.945

Pudzianowska, M., & Baird, J. H. (2021). Genetic diversity and species-specific DNA markers of Cynodon. Crop Science, csc2.20369. https://doi.org/10.1002/csc2.20369

Puechmaille, S. J. (2016). The program structure does not reliably recover the correct population structure when sampling is uneven: Subsampling and new estimators alleviate the problem. Molecular Ecology Resources, 16(3), 608–627. https://doi.org/10.1111/1755-0998.12512

Quinlan, A. R., & Hall, I. M. (2010). BEDTools: A flexible suite of utilities for comparing genomic features. Bioinformatics, 26(6), 841–842. https://doi.org/10.1093/bioinformatics/btq033

R Core Team. (2020). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. https://www.R-project.org

Raven, P. H. (2004). Taxonomy: Where are we now? Philosophical Transactions of the Royal Society B: Biological Sciences, 359(1444), 729–730.

Rouhan, G., & Gaudeul, M. (2014). Plant Taxonomy: A Historical Perspective, Current Challenges, and Perspectives. In P. Besse (Ed.), Molecular Plant Taxonomy: Methods and Protocols (pp. 1–37). Humana Press. https://doi.org/10.1007/978-1-62703-767-9_1

Sansaloni, C., Petroli, C., Jaccoud, D., Carling, J., Detering, F., Grattapaglia, D., & Kilian, A. (2011). Diversity Arrays Technology (DArT) and next-generation sequencing combined: Genome-wide, high throughput, highly informative genotyping for molecular breeding of Eucalyptus. BMC Proceedings, 5(7), Article 7. https://doi.org/10.1186/1753-6561-5-S7-P54

Semagn, K., Babu, R., Hearne, S., & Olsen, M. (2014). Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): Overview of the technology and its application in crop improvement. Molecular Breeding, 33(1), 1–14. https://doi.org/10.1007/s11032-013-9917-x

Semagn, K., Beyene, Y., Makumbi, D., Mugo, S., Prasanna, B. M., Magorokosho, C., & Atlin, G. (2012). Quality control genotyping for assessment of genetic identity and purity in diverse tropical maize inbred lines. Theoretical and Applied Genetics, 125(7), 1487–1501. https://doi.org/10.1007/s00122-012-1928-1

Sokal, R. R., & Sneath, P. H. (1963). Principles of numerical taxonomy. Principles of Numerical Taxonomy.

Taliaferro, C. M. (2003). Bermudagrass (Cynodon (L.) Rich). In Turfgrass Biology, Genetics, and Breeding (pp. 235–256). John Wiley & Sons.

Taliaferro, C. M., Hopkins, A. A., Henthorn, J. C., Murphy, C. D., & Edwards, R. M. (1997). Use of flow cytometry to estimate ploidy level in Cynodon species. International Turfgrass Society Research Journal, 8, 385–392.

Turland, N. J., Wiersema, J. H., Barrie, F. R., Greuter, W., Hawksworth, D. L., Herendeen, P. S., Knapp, S., Kusber, W.-H., Li, D.-Z., Marhold, K., May, T. W., McNeill, J., Monro, A. M., Prado, J., Price, M. J., & Smith, G. F. (2018). International Code of Nomenclature for algae, fungi, and plants (Shenzhen Code) adopted by the Nineteenth International Botanical Congress Shenzhen, China, July 2017. Koeltz Botanical Books. https://nhm.openrepository.com/handle/10141/622572

Vallejo-Marín, M., Cooley, A. M., Lee, M. Y., Folmer, M., McKain, M. R., & Puzey, J. R. (2016). Strongly asymmetric hybridization barriers shape the origin of a new polyploid species and its hybrid ancestor. American Journal of Botany, 103(7), 1272–1288. https://doi.org/10.3732/ajb.1500471

Vernesi, C., Crestanello, B., Pecchioli, E., Tartari, D., Caramelli, D., Hauffe, H., & Bertorelle, G. (2003). The genetic impact of demographic decline and reintroduction in the wild boar (Sus scrofa): A microsatellite analysis. Molecular Ecology, 12(3), 585–595. https://doi.org/10.1046/j.1365-294X.2003.01763.x

Wang, Z., Wu, Y., Martin, D. L., Gao, H., Samuels, T., & Tan, C. (2010). Identification of Vegetatively Propagated Turf Bermudagrass Cultivars Using Simple Sequence Repeat Markers. Crop Science, 50(5), 2103–2111. https://doi.org/10.2135/cropsci2010.02.0116

Weir, B. S., & Cockerham, C. C. (1984). Estimating F-Statistics for the Analysis of Population Structure. Evolution, 38(6), 1358–1370. https://doi.org/10.2307/2408641

Wieczorek, J. (2023). tdwg/dwc: 2023-07-10 (Version 2023-07-10) [Computer software]. Zenodo. https://doi.org/10.5281/zenodo.8132249

Wu, Y. Q., Taliaferro, C. M., Bai, G. H., Martin, D. L., Anderson, J. A., Anderson, M. P., & Edwards, R. M. (2006). Genetic Analyses of Chinese Cynodon Accessions by Flow Cytometry and AFLP Markers. Crop Science, 46(2), 917–926. https://doi.org/10.2135/cropsci2005.08.0256

Yang, L., Wu, Y., Moss, J. Q., Zhong, S., & Yang, B. (2018). Molecular Identification and Characterization of Seeded Turf Bermudagrass Cultivars Using Simple Sequence Repeat Markers. Agronomy Journal, 110(6), 2142–2150. https://doi.org/10.2134/agronj2018.01.0068

Zeisset, I., & Beebee, T. J. C. (2001). Determination of biogeographical range: An application of molecular phylogeography to the European pool frog Rana lessonae. Proceedings of the Royal Society of London. Series B: Biological Sciences, 268(1470), 933–938. https://doi.org/10.1098/rspb.2001.1600

Chapter 2

Responses of New Bermudagrass Genotypes to Prolonged and Recurrent Drought

Abstract

Increasing droughts coupled with decreasing available water resources in the Southwest highlight the need for new turfgrass cultivars that require less water. A field study evaluated a set of experimental and commercial intraspecific hybrid bermudagrasses [*Cynodon dactylon* (L.) Pers.] or interspecific (*C*. *dactylon* x *C. transvaalensis* Burtt Davy) for their responses to prolonged and repeated drought conditions in Riverside, CA (USDA Hardiness Zone 10a). Irrigation was withheld for two successive 60-d cycles of drought, each followed by 14-d recovery periods in 2020, 2021 and 2022. Plots were evaluated weekly for turf quality and leaf firing, as well as living green coverage, normalized difference vegetation index (NDVI), and dark green color index using digital imagery. Using living green coverage as a metric for drought performance, seven hybrids (Acc180012, Acc180037, Acc180040, Acc180146, Acc180217, Acc180229, Acc180557) were consistently among the top ten performers, on average retaining over 50% green coverage by the end of each dry-down cycle. In comparison, all control cultivars (Bandera, Riley's Super Sport (marketed as Celebration®), Santa Ana, TifTuf, Tifway II) had low to moderate responses, ranging from 36% to 0% green coverage. Celebration and TifTuf ranked the highest among control cultivars across years and cycles. Some accessions appeared to show stress memory, where the first drought period in a year appeared to prime them for a more successful second period each year. Results demonstrate extensive variation among

bermudagrass accessions in responses to drought and that evaluation over repeated drought cycles appears to be a useful selection tool for Mediterranean climates.

Introduction

Droughts have increased in both frequency and severity, posing major challenges to crop productivity and management on a global scale (Lobell and Gourdji, 2012). As a conservation measure, limitations have been imposed on the availability of water for urban irrigation, especially in the southwestern United States and other regions of similar climates. Warmer and drier climates in tandem with irrigation restrictions heavily impact turfgrasses and limit both their recreational and commercial facets. These conditions underscore the necessity for incorporating and improving drought resistant turfgrass cultivars and species, such as warm-season turfgrasses including bermudagrass (*Cynodon* spp.).

Drought stress manifests itself in plants via complex responses, impacting growth, development, and reproduction (Fang and Xiong 2015). Differences among turfgrasses are also apparent, especially between cool-season and warm-season species. In general, cool-season grasses are known to have higher water use rates than warm-season grasses based on their evapotranspiration (ET) rates (Huang and Fry 2000). The ET rates of cool-season grasses typically range between 4 to 13 mm per day, while warm-season grasses typically range from 3 to 9 mm per day (Huang 2008; Kenna 2008). Differences in water usage can be attributed to differences in functional traits, such as root and/or shoot characteristics. Variability in these traits is also present within cool-season and warm-season species. For example, when comparing multiple warm-season turfgrass species, Zhang et al. (2019) found that most differences in

drought resistance were due to differences in drought avoidance mechanisms, such as root depth and biomass, with bermudagrass showing the highest level of drought resistance (Zhang et al. 2019).

Bermudagrass has been well-studied for its higher natural resistance to drought conditions relative to other warm-season species. Bermudagrasses are able to avoid drought stress through increased rooting depth and density (Carrow 1995; Carrow 1996; Qian et al. 1997) and stomatal closure (Hu et al. 2009), enabling them to capture and conserve more water. Correlations between shoot characteristics and increased drought resistance have also been found for increased waxiness in cuticle layers (Zhou et al. 2014). At the biochemical level, studies in bermudagrass have shown higher drought tolerance to be associated with reduced proline content (Lu et al. 2009) and/or increased expression of dehydrins (Close 1997; Hu et al. 2010; Su et al. 2013), among other antioxidants. Cultural and management practices, such as fertilization or efficient irrigation, are additional facets that may influence drought resistance and are often overlooked or managed incorrectly by most consumers (Steinke et al. 2011).

With large genetic variability within the genus *Cynodon*, differences in drought resistance do exist among bermudagrass accessions (Shi et al. 2012; Zhou et al. 2013; Katuwal et al. 2022). Although bermudagrasses already demonstrate higher natural resistance to drought stress than other warm-season grasses, further improvement can be achieved by exploiting the natural genetic variation within the genus. Several studies have compared different commercial cultivars, with some comparing them against experimental lines in the context of selection in breeding programs. ‘Riley’s Super Sport’ (marketed as Celebration ®) Riley (2000) and ‘TifTuf’ (Schwartz et al. 2018), for example, have been observed to have high levels of drought resistance and are often

used as checks in drought response studies (Baldwin et al. 2006; Thapa 2011; Katuwal et al. 2020; Katuwal et al. 2022). Bermudagrasses and other warm-season grasses have also been evaluated for prolonged periods of drought in the field, showing a wide range of responses over 60 and 90 day droughts (Steinke et al. 2011; Severmutlu et al. 2011).

Although there have been studies highlighting the impressive drought resistance of warm-season turfgrasses, there have been no previous studies that evaluated their performance under repeated drought cycles, despite the known plasticity and adaptability of plants. One of the more interesting aspects of plant plasticity is 'stress memory'. The term describes a situation where a single stress event can prime a plant to respond differently to future stress conditions (Walter et al. 2011; Fan et al. 2020; Jacques et al. 2021). There have been few studies of this angle in turfgrass. It has been studied for recurrent heat stress in tall fescue (*Festuca arundinacea* Schreb.) (Hu et al. 2015; Bi et al. 2021), and for salinity stress in perennial ryegrass (Hu et al. 2016). However, in *Arabidopsis thaliana* recurrent drought and rehydration has been observed to induce transcriptional memory for stress-related genes, resulting in a slower wilting (Ding et al. 2012). The use of recurrent drought stress in bermudagrass has been previously alluded to by Zhang et al. (2019) and the effects of drought priming have been anecdotally observed from informal bermudagrass trials (Zhang et al. 2019).

This study was designed to evaluate intraspecific [*Cynodon dactylon* (L.) Pers.] and interspecific (*C*. *dactylon* x *C. transvaalensis* Burtt Davy) hybrids and commercial checks under prolonged, consecutive droughts covering the entire summer in a Mediterranean climate in Southern California. Screening by imposition of consecutive droughts, interspaced with short recovery periods, is tedious and long; however, it may more accurately reflect real-life conditions and identify germplasm able to cope better

with warming trends. Additionally, we aimed to identify how widely grown commercial cultivars compare in their response to stress priming. Stress memory is an important factor to developing new commercial varieties with consistent drought resistance across multiple years.

Methods

Plant material and experimental design

The experiment included 71 locally developed bermudagrass hybrids and five commercially available cultivars (Bandera (unpatented), Celebration (Riley 2000), Santa Ana (Youngner 1966), TifTuf (Schwartz et al. 2018), Tifway II (Burton 1981)) serving as checks. Two locally developed hybrid accessions, Presidio (Acc 17-8) (Baird et al. 2023a) and Coachella (Acc TP6-3) (Baird et al. 2023b), were patented after the conclusion of the experiment. Herein, these entries will be referred to by their original accession numbers. Collectively, the hybrids were derived from interspecific and intraspecific crosses among bermudagrass accessions. Crosses were either pair-wise, in which both parents were known, or from open-pollination of *Cynodon* spp. collection accessions, where only the maternal parent was known. In most cases, however, the hybrids were likely among accessions of *C. dactylon* or *C. dactylon* x *C. transvaalensis*. The checks were chosen to represent a cross section of the currently grown/recommended commercial cultivars of bermudagrass. Cores 6.25 cm in diameter were collected from field plots and planted in May 2019 to create 90-cm x 90-cm plots in three replicates (n = 228) under a completely randomized design (CRD) across two adjacent 27-m x 9-m fields at a field site on sandy loam. Of the 71 hybrids, six failed to

establish before the start of the experiment, or suffered a major setback in the early phases, and were removed. This left a total of 70 entries (5 checks and 65 hybrids) for evaluation (Table 1).

Throughout the growing season (prior to dry-down initiation), irrigation was supplied by sprinklers three times per week at 100% of the monthly reference evapotranspiration rate. The plots were mowed at 1.25 cm three times per week. Plots received 15 g m^{-2} yr^{-1} in May using controlled release urea (41-0-0). Glyphosate was used to control plot growth and maintain plot dimensions. No fertilizer or herbicide was applied during the dry-down or recovery phases of the experiments. Natural rainfall is rare during the summer months in the region, hence the amounts of water provided to test plots usually can be fully controlled. Fortuitously, substantial rainfall in September 2022 coincided with the start of the second recovery period at the end of the experiment (Figure 2.1) and is not believed to have significantly affected the results.

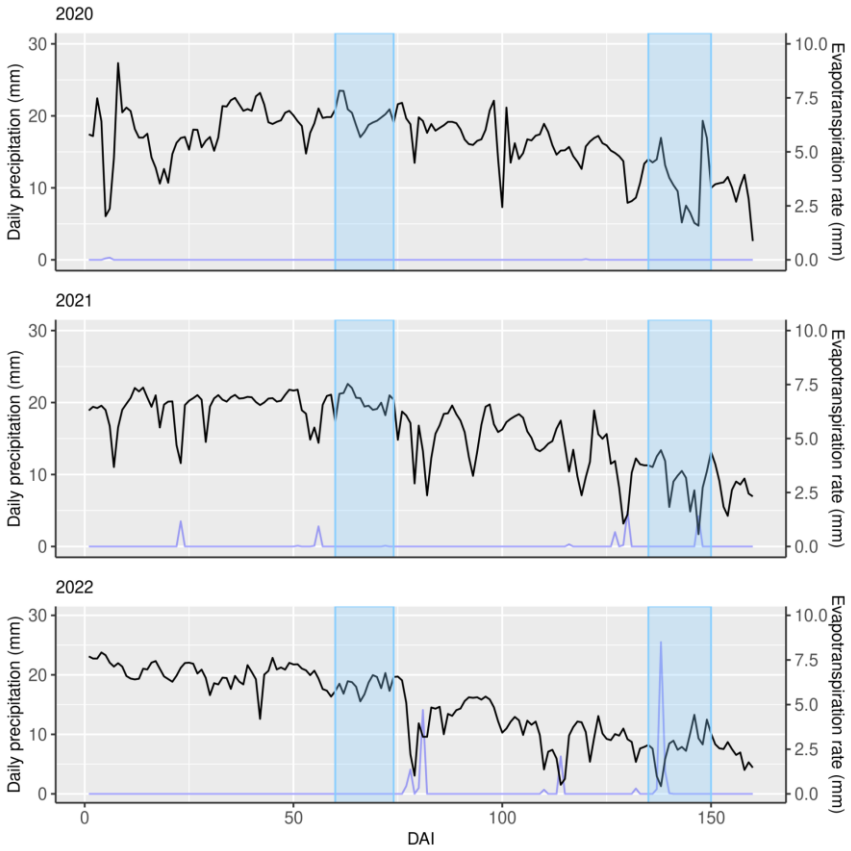

Figure 2.1 Daily precipitation and evapotranspiration rates for each dry-down study from 2020, 2021, and 2022. Blue shaded regions represent recovery periods for each year. Blue lines represent daily precipitation (mm), while black lines represent average daily evapotranspiration (mm). DAI = Days after study initiation. The dry-down began on June 1 for both 2020 and 2021, and June 22 for 2022.

Table 2.1 List of bermudagrass genotypes and their origin used in dry-down experiments.

Genotype	Type of entry	Genotype	Type of entry	Genotype	Type of entry
Bandera	Cultivar	Acc180040	Local BL	Acc180551	Local BL
Celebration	Cultivar	Acc180044	Local BL	Acc180555	Local BL
Santa Ana	Cultivar	Acc180049	Local BL	Acc180557	Local BL
TifTuf	Cultivar	Acc180077	Local BL	Acc180572	Local BL
Tifway II	Cultivar	Acc180118	Local BL	Acc180575	Local BL
Acc 10-9	Local BL[a]	Acc180120	Local BL	Acc180576	Local BL
Acc 17-8 (Presidio)	Cultivar	Acc180127	Local BL	Acc180578	Local BL
Acc 5-8	Local BL	Acc180128	Local BL	Acc180579	Local BL
Acc BF1	Local BL	Acc180133	Local BL	Acc180580	Local BL
Acc BF2	Local BL	Acc180146	Local BL	Acc180581	Local BL
Acc CVARS1	Local BL	Acc180164	Local BL	Acc180583	Local BL
Acc CVARS2	Local BL	Acc180173	Local BL	Acc180585	Local BL
Acc CVARS3	Local BL	Acc180174	Local BL	Acc180589	Local BL
Acc TP3-2	Local BL	Acc180175	Local BL	Acc180592	Local BL
Acc TP4-1	Local BL	Acc180200	Local BL	Acc180594	Local BL
Acc TP4-2	Local BL	Acc180203	Local BL	Acc180602	Local BL
Acc TP6-3 (Coachella)	Cultivar	Acc180211	Local BL	Acc180603	Local BL
Acc180009	Local BL	Acc180215	Local BL	Acc180640	Local BL
Acc180012	Local BL	Acc180217	Local BL	Acc180659	Local BL
Acc180014	Local BL	Acc180220	Local BL	Acc180668	Local BL
Acc180015	Local BL	Acc180229	Local BL	Acc180681	Local BL
Acc180024	Local BL	Acc180247	Local BL	Acc180724	Local BL
Acc180037	Local BL	Acc180473	Local BL		
Acc180038	Local BL	Acc180549	Local BL		

[a] Local BL = Local Breeding Line

All plots were evaluated for their responses to prolonged drought stress during Southern California summers, from June to November for three years, from 2020 to 2022. Each year, the plots underwent an initial water saturation period where 150% of the previous week's short grass reference evapotranspiration rate (ET_{os}) was replaced via irrigation for 14 days. Irrigation was based on data from the California Irrigation Management System (CIMIS) weather station located on tall fescue turf approximately 200 m from the study area (U.C. Riverside #44). Daily weather data such as evapotranspiration, precipitation, and high and low temperatures were also compiled from the CIMIS weather station. This was followed by two consecutive dry-down periods, where no irrigation was supplied (0% ET_{os}), each followed by a 14-day recovery period (150% ET_{os}). In 2020, dry-down periods were 64 days long, while recovery periods were 14 days long (156 days total). In 2021 and 2022, dry-down periods were 60-days long, while the recovery periods remained at 14 days (148 days total).

Data collection

Plots were evaluated weekly around solar noon using both subjective and objective criteria. Each plot was given a visual score for turf quality (VQ; 1 to 9, 9 = best) and leaf firing (LF; 1 to 9, 9 = no firing). Each plot was also measured for percent green coverage (%GC; 0% to 100%), normalized difference vegetative index (NDVI; 0.00 to 1.00), and dark green color index (DGCI). NDVI is a general plant health indicator based on the absorbance and reflectance of red and infrared light, respectively, by a handheld sensor (GreenSeeker; Trimble Inc., Westminster, CO, USA). A metal lightbox (0.61-m x 0.51-m x 0.56-m) with a mounted digital camera was used to photograph each plot

under consistent conditions (Karcher and Richardson 2013). Images were then subsequently processed with digital image analysis using the Turf Analyzer software (Karcher et al. 2017) to measure the %GC and DGCI for each plot.

Statistical analysis

The recorded measurements for each genotype were analyzed in RStudio [ver 2023.06.0+421] (RStudio Team 2020). Data were analyzed for each year and each cycle per year with linear mixed models with repeated measures using the 'nlme' package in R (Pinheiro et al. 2017). Genotype and dry-down cycles were considered as fixed effects, and individual plants and measurement dates as random effects. As responses were measured for two dry-down cycles per year, data from each cycle were analyzed separately. The effects of genotypes, cycles, and their interactions were tested for significance (F-test). Genotype means were separated by Fisher's protected least significant difference (LSD) test. Although this generates many comparisons and increases the potential for Type I error, our goal was to make comparisons between dry-down cycles while avoiding Type II errors and has been demonstrated to be an effective approach (Saville 2015; Robins and Bushman 2020).

Percent green coverage was plotted against cumulative evapotranspiration (ETcum) and day of dry-down cycle (DoC) under two separate models, and a strong nonlinear relationship was observed. The following equation was used to model the data:

$$\%GC = \frac{d}{1 + exp\big(-b(x - e)\big)}$$

where *d* is the maximum percent green coverage for a genotype at the start of a dry-down cycle, *X* is the predictor variable (ETcum or DoC), and *b* and *e* are estimated model parameters corresponding to the slope and GC50, respectively. The variable *d* was set to the upper limit of 100.00 to maintain biological significance. The slope (*b*) parameter defines how quickly the percent green coverage of a genotype declines over time. GC50 (*e*) is estimated to be the value of *X* when the percent green coverage of a genotype reaches 50% of the initial value (*d*). Nonlinear regression analysis was performed for each genotype using the 'drc' and 'aomisc' packages in R (Ritz et al. 2016; Onofri 2020). Multiple regression models were developed separately for each accession in each dry-down cycle. The Akaike information criterion (AIC) was used to compare the ETcum and DoC models. To measure the goodness-of-fit for our models, the pseudo R^2 value was examined. The pseudo R^2 value can be viewed as an analogue of the coefficient of determination (R^2) used in linear regression, and offers a more intuitive value than then Mean Squared Error (MSE) or the AIC and Bayesian information criterion (BIC) (Pierce 2001; Spiess and Neumeyer 2010).

Results

Daily climate conditions were noted throughout each dry-down cycle. The first dry-down cycle had a higher cumulative evapotranspiration rate (392.30-mm, 387.02-mm, 405.59-mm) than the second cycle (334.29-mm, 292.74-mm, 228.87-mm) for 2020, 2021, and 2022, respectively. The conditions in 2022 were warmer and drier than in

2020 or 2021, and may have contributed to the observed response variation. The average daily evapotranspiration rate was higher and more consistent during the first dry-down cycle of 2022 (6.76 mm) than in 2020 (6.13-mm) or 2021 (6.45 mm), but lower in the second cycle of 2022 (3.75 mm) in comparison with 2020 (5.22-mm) and 2021 (4.80-mm). Average daily maximum and minimum temperatures increased each year from 2020 to 2022 during the first cycle, but decreased each year during the second cycle (Figure 2.2). Total precipitation was recorded across each study year, with higher amounts recorded during 2021 (13.9 mm) and 2022 (28.4 mm). Only 0.6 mm of total precipitation was recorded in 2020. Unusually high rainfall in 2022 coincided with the second recovery period and does not seem to have affected the results (Figure 2.2).

The dry-down cycles in 2020 (64 days) were longer than those in 2021 and 2022 (60 days). This was done in expectation that additional four days would amplify differences among the accessions. However, as no such amplification was observed, the 60-day period was deemed sufficient to observe phenotypic separation among the accessions without jeopardizing plot survival and leaving sufficient time to complete the second dry-down cycle before autumn rains.

Overall, a very wide range of responses was observed, both among the hybrids and the checks. These ranged from minimal declines in green coverage in both dry-down cycles followed by rapid recoveries, to dramatic declines followed by poor recoveries (Figure 2.3; Table 2.2-3, Supplemental Table S2.1). Due to the large number of accessions, %GC was presented for only 14 accessions (seven cultivars and seven top performing hybrids across all years) (Table 2.2-3). Tables for all entries and traits can be found in the Supplemental Tables S2.1-5. Comparisons were made between the

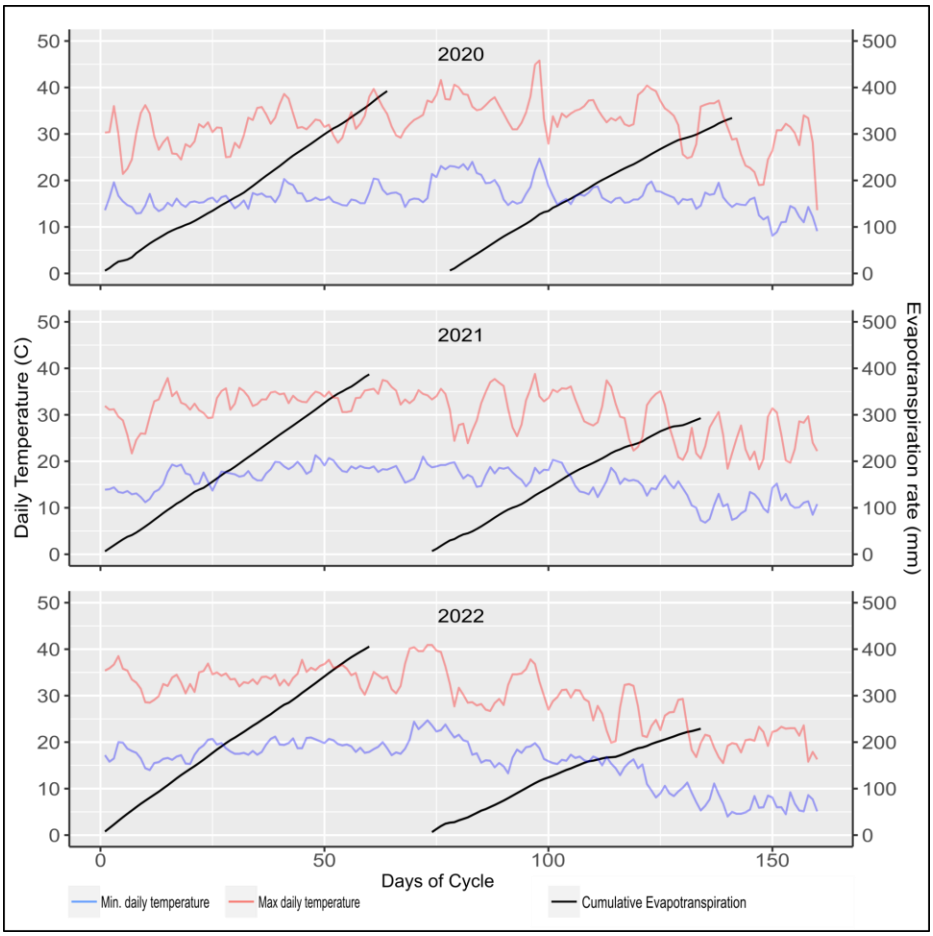

Figure 2.2 Maximum and minimum daily temperatures as well as cumulative evapotranspiration rates for each dry-down study in 2020, 2021, and 2022. Daily temperatures and average evapotranspiration rates were recorded from the California Irrigation Management Information System (CIMIS, U.C. Riverside station #44). Maximum and minimum daily temperatures are represented by red and blue lines, respectively. Cumulative evapotranspiration (black) was calculated for each drought cycle, but not for recovery periods due to the reintroduction of irrigation during those times.

first and second cycle each year. The range of responses to the first and second dry-down cycles were quite similar within each year, but more so in 2020 vs. 2021, where the %GC on the final day ranged from full dormancy to about 90% for both cycles. In 2022, the %GC on the final day ranged from full dormancy to 42% GC in the first cycle, and full dormancy to 66% GC in the second cycle. Comparisons were also made between the first and the second cycle of each year. The difference between the start

and end values of each cycle was calculated and expressed as a percentage (% change over drought). The average % change for the first cycle each year was -43.39%, -63.54%, and -93.08% for 2020, 2021, and 2022, respectively (Table 2.2). For the second cycle each year, the average % change over drought was -51.51%, -43.54%, and -84.24% for 2020, 2021, and 2022, respectively (Table 2.3). Each year, a narrow range of recovery responses was observed following the first dry-down cycle, where most accessions were able to recover close to their initial values, indicating that most accessions were able to manage an initial 60-day drought reasonably well. However, a much wider range of recovery responses was observed in the second recovery period for each year, indicating minimal recovery for most accessions. On average, accessions recovered an additional 56% GC by the end of the first recovery period, but only an additional 20% GC by the end of the second. For example, if an accession started the first recovery period with 25% GC, then it would recover to 76% GC by the end of the recovery period. If an accession started the second recovery period with 25% GC, then it would recover to 45% GC.

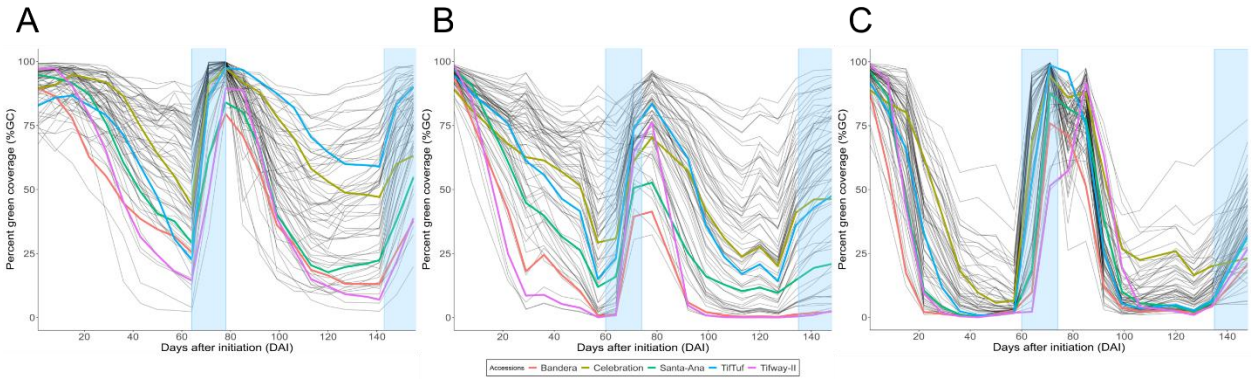

Figure 2.3 Comparison of mean green cover percentage between commercial standards and hybrids. Each line represents the mean green coverage across 2020 (A), 2021 (B), and 2022 (C) for a single accession. Blue regions represent recovery periods (14-days). Hybrids are shown by black lines.

Table 2.2 Comparison of percent green coverage at three time points each year during the first dry-down cycle for cultivars and seven top performing bermudagrass entries.

Genotype	Start of cycle 1	End of cycle 1[a]	End of recovery 1	Change over drought
	——————————%GC——————————			%
	2020			
Bandera	89.55	25.53	79.49	-71.50
Acc TP6-3 (Coachella)	97.01	24.75	97.90	-74.49
Acc 17-8 (Presidio)	96.12	20.86	95.72	-78.30
Celebration	89.54	43.95	97.44	-50.92
Santa Ana	94.77	29.18	83.99	-69.21
TifTuf	82.75	22.59	97.44	-72.70
Tifway-II	96.94	14.46	89.45	-85.09
Acc180012	90.71	83.32	97.59	-8.15
Acc180037	84.28	78.48	95.28	-6.88
Acc180040	95.20	90.06	98.08	-5.41
Acc180146	95.66	69.32	99.78	-27.54
Acc180217	73.94	78.01	94.52	5.51
Acc180229	89.11	83.96	99.66	-5.78
Acc180557	95.53	87.11	99.32	-8.82
	2021			
Bandera	94.23	0.58	39.44	-99.38
Acc TP6-3 (Coachella)	93.99	2.72	61.40	-97.11
Acc 17-8 (Presidio)	98.30	0.92	48.51	-99.06
Celebration	89.01	29.30	61.12	-67.08
Santa Ana	96.87	11.96	50.59	-87.66
TifTuf	94.53	14.98	74.06	-84.16
Tifway-II	98.46	0.08	64.55	-99.92
Acc180012	94.99	78.64	79.97	-17.22
Acc180037	92.01	72.70	67.51	-20.98
Acc180040	98.46	82.70	86.11	-16.00
Acc180146	97.94	86.59	88.13	-11.59
Acc180217	91.95	76.55	83.93	-16.75
Acc180229	94.74	66.82	76.99	-29.47
Acc180557	96.01	90.46	77.45	-5.78
	2022			
Bandera	87.59	2.66	75.93	-96.96
Acc TP6-3 (Coachella)	97.11	2.45	59.15	-97.47
Acc 17-8 (Presidio)	95.94	1.86	56.85	-98.06
Celebration	88.90	6.56	94.39	-92.62
Santa Ana	96.35	1.95	87.63	-97.97
TifTuf	91.72	2.05	98.71	-97.77
Tifway-II	98.76	1.86	51.43	-98.11
Acc180012	87.76	33.86	99.15	-61.42
Acc180037	94.70	22.27	96.28	-76.48
Acc180040	97.08	21.47	99.07	-77.88
Acc180146	99.20	17.65	99.54	-82.21
Acc180217	95.37	23.34	97.76	-75.53
Acc180229	96.70	10.50	98.51	-89.14
Acc180557	94.41	42.11	98.18	-55.40

Abbreviations: %GC, Percent green cover

[a] "End of cycle 1" is the same as "Start of recovery 1"; recovery irrigation was initiated on the same day

Table 2.3 Comparison of percent green coverage at three time points each year during the second dry-down cycle for cultivars and seven top performing bermudagrass entries.

Genotype	Start of cycle 2	End of cycle 2[a]	End of recovery 2	Change over drought
	——————————%GC——————————			%
	2020			
Bandera	79.49	13.12	37.68	-83.50
Acc TP6-3 (Coachella)	97.90	31.75	66.92	-67.57
Acc 17-8 (Presidio)	95.72	19.12	68.71	-80.02
Celebration	97.44	47.04	63.15	-51.73
Santa Ana	83.99	22.39	54.70	-73.35
TifTuf	97.44	58.94	90.11	-39.51
Tifway-II	89.45	7.00	38.78	-92.18
Acc180012	97.59	82.58	94.60	-15.38
Acc180037	95.28	80.98	85.24	-15.00
Acc180040	98.08	94.69	97.37	-3.45
Acc180146	99.78	91.68	98.46	-8.12
Acc180217	94.52	81.47	83.50	-13.80
Acc180229	99.66	82.82	90.12	-16.89
Acc180557	99.32	89.44	94.34	-9.95
	2021			
Bandera	39.44	1.25	2.20	-96.84
Acc TP6-3 (Coachella)	61.40	19.18	26.72	-68.76
Acc 17-8 (Presidio)	48.51	5.50	7.88	-88.66
Celebration	61.12	40.92	46.24	-33.05
Santa Ana	50.59	14.62	20.90	-71.11
TifTuf	74.06	36.03	47.51	-51.35
Tifway-II	64.55	0.37	2.58	-99.43
Acc180012	79.97	86.65	90.05	8.35
Acc180037	67.51	65.42	72.32	-3.09
Acc180040	86.11	87.45	89.73	1.56
Acc180146	88.13	89.68	94.67	1.76
Acc180217	83.93	80.88	90.98	-3.64
Acc180229	76.99	67.15	78.20	-12.78
Acc180557	77.45	93.27	96.86	20.42
	2022			
Bandera	75.93	4.16	21.00	-94.52
Acc TP6-3 (Coachella)	59.15	4.75	18.59	-91.97
Acc 17-8 (Presidio)	56.85	4.24	20.72	-92.55
Celebration	94.39	20.04	23.07	-78.77
Santa Ana	87.63	5.55	31.52	-93.66
TifTuf	98.71	6.46	32.09	-93.46
Tifway-II	51.43	4.57	26.87	-91.11
Acc180012	99.15	39.86	43.37	-59.80
Acc180037	96.28	22.06	27.32	-77.08
Acc180040	99.07	23.47	13.01	-76.31
Acc180146	99.54	29.33	44.34	-70.54
Acc180217	97.76	30.17	52.86	-69.14
Acc180229	98.51	24.68	38.20	-74.95
Acc180557	98.18	65.99	77.43	-32.79

Abbreviations: %GC, Percent green cover

[a] "End of cycle 2" is the same as "Start of recovery 2"; recovery irrigation was initiated on the same day

Similar patterns were observed for NDVI values (Supplemental Table S2.2). In 2020 and 2021, NDVI values ranged from 0.19 to 0.64 by the end of the first dry-down cycle but ranged from 0.11 to 0.41 for 2022. By the end of the second dry-down cycle, NDVI ranged from 0.23 to 0.67 in 2020, 0.16 to 0.60 in 2021, and 0.15 to 0.50 in 2022. Most accessions were able to recover to unstressed levels (NDVI > 0.50) by the end of the first recovery period in the first two years, but no accessions were able to recover to unstressed levels in the third year. In 2020, most plants were able to recover to unstressed levels by the end of the second recovery period; in 2021 and 2022 most plants continued to exhibit moderate levels of stress or poor health.

The five checks evaluated in this study consistently occupied the bottom half of the range of responses across years. Celebration and TifTuf ranked the highest among the five across years and cycles, with Celebration retaining the highest mean %GC at the end of both the first (43.95%, 29.30%, 6.56%) and the second (47.04%, 40.92%, 20.04%) dry-down cycles for 2020, 2021, and 2022, respectively. TifTuf had the highest recovery potential measured by %GC at the end of the first (97.44%, 74.06%, 98.71%) and second (90.11%, 47.51%, 32.09%) recovery periods in 2020, 2021, and 2022, respectively. These results were also paralleled by NDVI, VQ, LF, and DGCI (Supplemental Table S2.2-5). Bandera and Tifway II performed similarly, but were ranked the lowest among the five checks, with %GC values less than 25% at the end of the first cycle in 2020 and less than 10% in 2021 and 2022. Values for %GC for Bandera and Tifway II were less than 15% at the end of the second cycle for all three years. Among the hybrids, Acc180557 held the top rank across years with the highest mean %GC at the end of both the first (87.11%, 90.46%, 42.11%) and second (89.44%, 93.27%, 65.99%) dry-down cycles in 2020, 2021, and 2022, respectively. Other high

performing hybrids (Acc180012, Acc180037, Acc180040, Acc180146, Acc180217, Acc180229) also behaved consistently across years. The seven listed hybrids remained among the top ten best performers each year for both dry-down cycles based on their %GC.

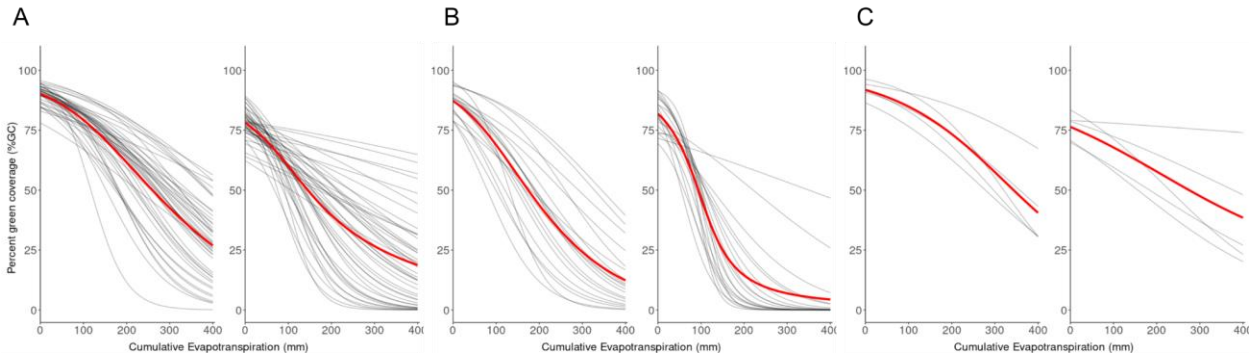

Figure 2.4 Determining stress memory types based on a comparison of the change in slope between the first and second dry-down cycles across years. The dry-down curve of each genotype (black) was compared between the first and second dry-down cycles across years. For A, B, and C, the left graph shows the curve during the first dry-down, while the right graph shows the curve during the second dry-down. If the difference between the slopes of these curves did not increase or decrease by more than 33%, they were assigned as 'neutral' types (A). Entries where the change in the slope of the curve decreased by more than 33%, were assigned as 'susceptible' types (B). Entries where the change in the slope increased by more than 33% were assigned as 'resistant' types (C). A curve was fitted for the mean response of all genotypes for each stress memory type (red) to better visualize the change in the slope between cycles.

To test for the possible stress memory among the genotypes, the rate of change in response (slope) was derived from the nonlinear regression models for each genotype. For each genotype, the slope of the curve was compared between the first and second dry-down cycles (Figure 2.4). A change between the slopes by 33% was taken as a threshold based on visual inspection of the regression models. This was done to group the genotypes into one of three observed stress memory types: 1) "neutral", where slopes were similar between both dry-down cycles; 2) "susceptible", where the rate of decline was larger (steeper) in the second dry-down cycle; and 3) "resistant",

where the rate of decline was smaller (shallower) in the second dry-down cycle (Figure 2.4). The division of genotypes into three groups, by 33%, is arbitrary. To take into account variability in annual climate conditions groupings were based on similar percent changes in the slopes between at least two years. In this manner, 45 genotypes were assigned as “neutral”, 20 genotypes as “susceptible”, and five genotypes as “resistant” types (Table S2.6).

Differences in response trends were evident between genotypes, and individual nonlinear regression models were found to be more appropriate over a global model in describing the responses of each genotype. Individual nonlinear regression models for each genotype and the dry-down cycle, paired with the linear mixed model, appeared to offer the best insight into the genetic differences between individual genotypes (Thapa 2011; Katuwal et al. 2022). Additionally, individual models reduce errors that might arise from the pooled variance, which is a feature of any global model. Comparisons were made between the nonlinear regression models using ETcum or DoC as the predictor. In both cases, the data fit well with a sigmoid variable slope model, as seen in similar drought studies (Karcher et al. 2008; Katuwal et al. 2022). The AIC value was lower overall in models that were based on cumulative evapotranspiration, prompting us to select ETcum as the predictor for further analyses. Based on the data, the pseudo R^2 values show a wide range of how well the models fit the data in both dry-down cycles. In the first dry-down cycle, pseudo R^2 ranged from 0.20 to 0.86. In the second dry-down cycle, pseudo R^2 ranged from 0.01 to 0.85. Genotypes where the nonlinear regression model fit poorly were typically the high-performing entries. For example, Acc180557 had the pseudo R^2 values of 0.29 and 0.01 for the first and second dry-down cycles, respectively. Given the slow decline in performance for Acc180557, we believe that

longer drought cycles would be needed to properly fit a model explaining its performance under drought. The AIC values supported the decision to develop individual models, and pseudo R^2 values demonstrated the goodness-of-fit for using a parsimonious regression model based on the three-parameter logistic model. Pseudo R^2 is recommended over the R^2, the latter of which is typically used for linear regression (Pierce 2001; Spiess and Neumeyer 2010).

Correlation analysis was performed for each dry-down cycle. Drought response traits (VQ, LF, %GC, NDVI, DGCI) had a strong positive correlation with each other, with Spearman coefficients (ρ) ranging from ρ = 0.664 to 0.942 in 2020, ρ = 0.826 to 0.955 in 2021, and ρ = 0.634 to 0.952 in 2022 for the first dry-down cycle. Spearman coefficients ranged from ρ = 0.777 to 0.970 in 2020, ρ = 0.747 to 0.960 in 2021, and ρ = 0.583 to 0.932 in 2022 for the second dry-down cycle. For all years and cycles, the correlations between %GC and NDVI and between %GC and VQ were the strongest, with Spearman coefficients greater than 0.9. There was also a strong positive correlation between VQ and NDVI for each cycle and year, ranging from ρ = 0.839 to 0.946. These findings align with previous field studies that have suggested NDVI may be a useful metric for drought responses, given that VQ is subjective by nature and takes into account other plant features (Katuwal et al. 2022). Moderate to strong negative correlations were observed between each of the drought response traits and the predictors (DoC, ETcum), ranging from ρ = -0.464 to -0.803 in 2020, ρ = -0.500 to -0.722 in 2021, and ρ = -0.635 to -0.778 in 2022 for the first dry-down cycle. Spearman coefficients ranged from ρ = -0.465 to -0.623 in 2020, ρ = -0.458 to -0.565 in 2021, and ρ = -0.658 to -0.755 in 2022 for the second dry-down cycle. The predictors, DoC and ETcum, showed a nearly monotonic relationship in each dry-down cycle. The ETcum had a slightly higher correlation with

response variables than DoC, supporting its use in our models. It should be noted, however, that the recorded cumulative evapotranspiration values are based on CIMIS data using a reference plot under no stress. True values likely vary among genotypes due to differences in drought avoidance traits (e.g. rooting) that stem from genetic effects (Zhou et al. 2009). All drought response traits and predictors were significantly correlated ($p < 0.001$) with each other for both dry-down cycles (Figure 2.5).

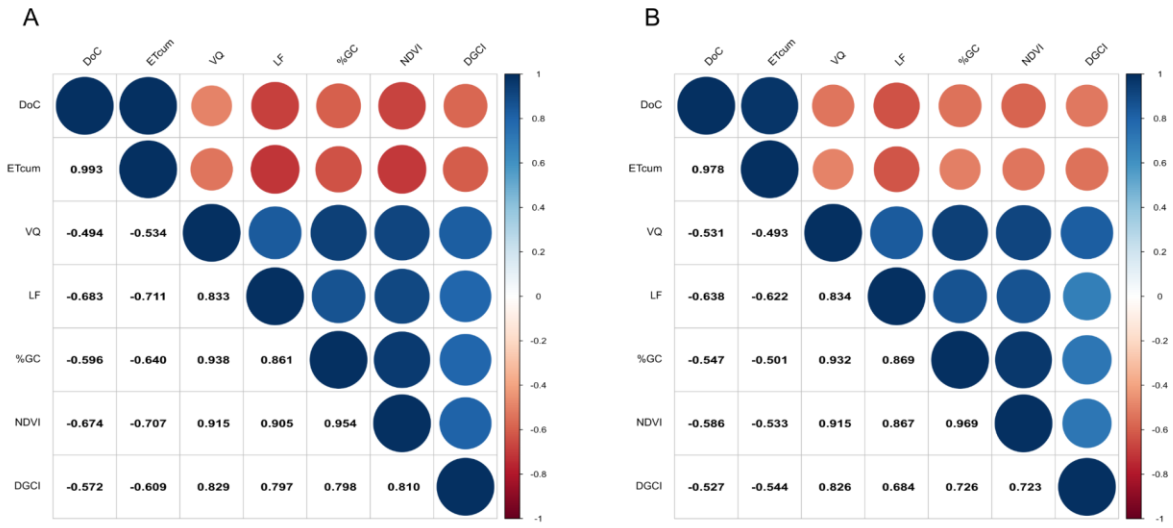

Figure 2.5 Spearman rank correlation between response traits and predictors for each dry-down cycle. A & B represent the first and second dry-down cycles, respectively. Numbers in the lower triangle are the correlation coefficients. Circle size in the upper triangle corresponds with the absolute values of the correlation coefficients, and color intensity corresponds with a positive (blue) or negative (red) correlation. Response traits: VQ = visual quality, LF = leaf firing, %GC = percent green coverage, NDVI = Normalized Difference Vegetation Index, DGCI = dark green color index. Predictors: DoC = day of (dry-down) cycle, cumET = cumulative evapotranspiration. All correlations were significant at $P < 0.001$. No correlations were found to be statistically not significant (ns).

Discussion

Rising global temperatures have posed many challenges to the turfgrass industry, resulting in the call for more drought-resistant cultivars. Selection for improved

drought resistance across all turfgrass species has primarily relied on measuring shoot and/or root performance during brief drought conditions. Cultivars produced in this manner have been successful, but with current global climate trends, more intensive selection criteria may be required, to reflect the increasing frequency and severity of droughts. This is especially important when considering the payback period, in which consumers will see returns on investment through water savings when investing in new commercial varieties (Minor et al. 2020). In this study, recurrent prolonged dry-down cycles illustrated differences in response patterns to stress, including a potential priming effect that appears to be dependent on the genetic background. Knowledge of how different genotypes respond to repeated drought stress conditions should be important to turfgrass breeders as it opens to further discussion and experimentation regarding water management practices and is cognizant of the potential for increased restrictions on irrigation water in the future.

Considerable variation in responses were observed for every character scored and measured, and created a challenge at analyses. For example, single dry-down experiments for bermudagrass are typically conducted during the summer months of June through September, but given the prolonged nature of this study, data were collected outside of the summer season and may explain some of the variability observed. This means that the first dry-down each year was an evaluation of plant responses to both heat and drought stress, while the second dry-down each year mostly evaluated drought stress alone. Lower temperatures coincided with the second recovery period in all years. Chilling stress for warm-season grasses occurs between 0℃ and 15℃, making it likely that the lower temperatures contributed to slower growth and recovery after the second dry-down cycle (Levitt 1980). A large decline in the average

performance following the first dry-down was observed across study years, with a marked difference between the first and last study years. These results did not align with cumulative evapotranspiration rates (392.30-mm, 387.02-mm, 405.59-mm for 2020, 2021, and 2022, respectively) and signal that stress priming did not persist from year to year as we would expect. One explanation might be carbohydrate depletion (Heji et al. 2016; Zhang et al. 2019), which would be further compounded by experiencing two dry-down cycles per year. Enhanced root development, as a means of drought avoidance, does not appear to be a major factor in this study. Such drought-induced enhanced growth should have carried on from the first to the second drought period each year, and from one year to the next (assuming no significant decrease in root biomass during dormancy). Based on these results, tolerance is more likely to be enhanced through priming via induction of genes related to homeostasis and reduction of oxidative stress. Another explanation might be the prolonged duration of each dry-down cycle. Previous drought stress priming studies in other plant species have typically used short stress periods no longer than 30 days. It is possible that there is an innate threshold for each genotype where, much like a rubber band, if the threshold is exceeded then the rubber band breaks and priming does not persist, leading the plant to focus on survival.

The percentage of living green coverage was used as the main trait for drought response in this study. Although “turf quality” is widely used, in research and breeding , it is a subjective measure based on color, density, uniformity, texture, and the transient effects of environmental stresses that may not be entirely indicative of drought response (Morris and Shearman 1998). In recent years, the adoption of high-throughput phenotyping methods in breeding programs has introduced objective methods, including digital image analysis, spectral reflectance, and unmanned aerial vehicles (UAVs) for

data collection, and offers more objective and comparable data between different environments and years (Yang et al. 2017; Herzig et al. 2021).

The assignment of individual entries to one of the three stress memory types was arbitrarily based on the percent change in the regression line slopes between dry-down cycles. In fact, the variation in performance appeared to be continuous (Figure 2.3). Accessions classified as ‘neutral’ types would suggest an absence of a priming effect to drought stress. ‘Susceptible’ types, however, may have a mechanism to recognize recurrent stress and may attempt to avoid it by initiating genes associated with drought escape, or dormancy induction. ‘Resistant’ types may have a similar recognition mechanism but can enhance drought tolerance or avoidance by initiating different subsets of stress-response genes (Fan et al. 2020). Under recurring drought/dehydration, increases in transcript levels of stress-response genes were observed for *Arabidopsis* plants when compared to levels during the initial drought stress, demonstrating the presence of a transcriptional memory (Ding et al. 2012; Ding et al. 2013). Four types of stress memory genes were identified, with each type contributing to either drought escape or drought tolerance/avoidance. Bermudagrass may well utilize a similar mechanism to prime for drought escape or drought tolerance/avoidance and would support the idea of three stress memory classes based on phenotype. Additional studies would be required to confirm that the phenotypic stress memory types observed in our study correspond with differences in transcriptional responses.

Strictly controlled environmental conditions, such as growth chambers, would have been better for some aspects of this study, such as the possible priming effect. For one, this would have avoided the possible confounding effect of heat stress during the

first drought-recovery cycle, and lower temperatures during the second recovery periods, and allowed more than just two cycles. In the field, variability in climate conditions between years was observed to be more dramatic than expected, with temperatures steadily rising from year to year during the first dry-down cycle, and declining year to year during the second dry-down cycle. However, the idea was to evaluate a wide range of germplasm under real life conditions, also meant as a tool in breeding. The results clearly show a wide range of drought responses among the entries. Although it would have been ideal, we were unable to measure avoidance traits (such as root depth and distribution or shoot growth patterns) or tolerance mechanisms (such as protein or metabolite accumulation). Measurements of these traits would have provided additional context to better understand the potential for priming in this study. Despite these shortcomings, we believe that this study provides the first steps to understanding drought stress priming through the observation of different stress response types.

Stress priming has not been well-explored in the context of breeding or irrigation management. Although the best performers are often sought out in breeding programs based on their mean response values, the addition of modeling these responses can provide insight and a better context for selection decisions. For example, the best performer(s) were identified here based on the percentage of living green color, but may have differences in certain quality-related traits (e.g. genetic color, texture, etc.). Accessions can be selected that strike a balance between high quality ratings and high or moderate drought resistance based on the number of days of drought and the expected living green coverage from models. Similarly, models in this context can be used to evaluate cultivars and provide turf managers with better insight on how to irrigate them.

In an effort to demonstrate the applications of selection via recurrent dry-downs, the best performing hybrid, Acc180557, was compared to the best among five commercial checks, TifTuf, using %GC as the main criterion (Figure 2.6). TifTuf has been reported to have high drought tolerance and superior visual quality (Minor et al. 2020). In this experiment, TifTuf performed the best among the five commercial cultivars used as checks but showed a dramatic decrease in green coverage across each year during both dry-down cycles, recovering to only about one half of the initial green coverage by the end of each study year. Leaf firing rapidly increased in severity for TifTuf around two weeks after the start of each dry-down cycle, while VQ, NDVI, and DGCI declined more steadily. Acc180557 showed a mild response to drought stress across each year during both dry-down cycles, while making a nearly full recovery to the initial green coverage by the end of each study year. Other measured responses followed a similar trend of slow decline over the course of each dry-down cycle. TifTuf did, however, outperform Acc180557 for DGCI after the end of the first recovery period. TifTuf appeared to have a ‘neutral’ stress memory type, where the rate of decline did not substantially change from the first to the second dry-down cycles, while Acc180557 appears to have a ‘resistant’ stress memory type, as its rate of decline in the second dry-down period was shallower after priming in the first dry-down cycle (Figure 2.6).

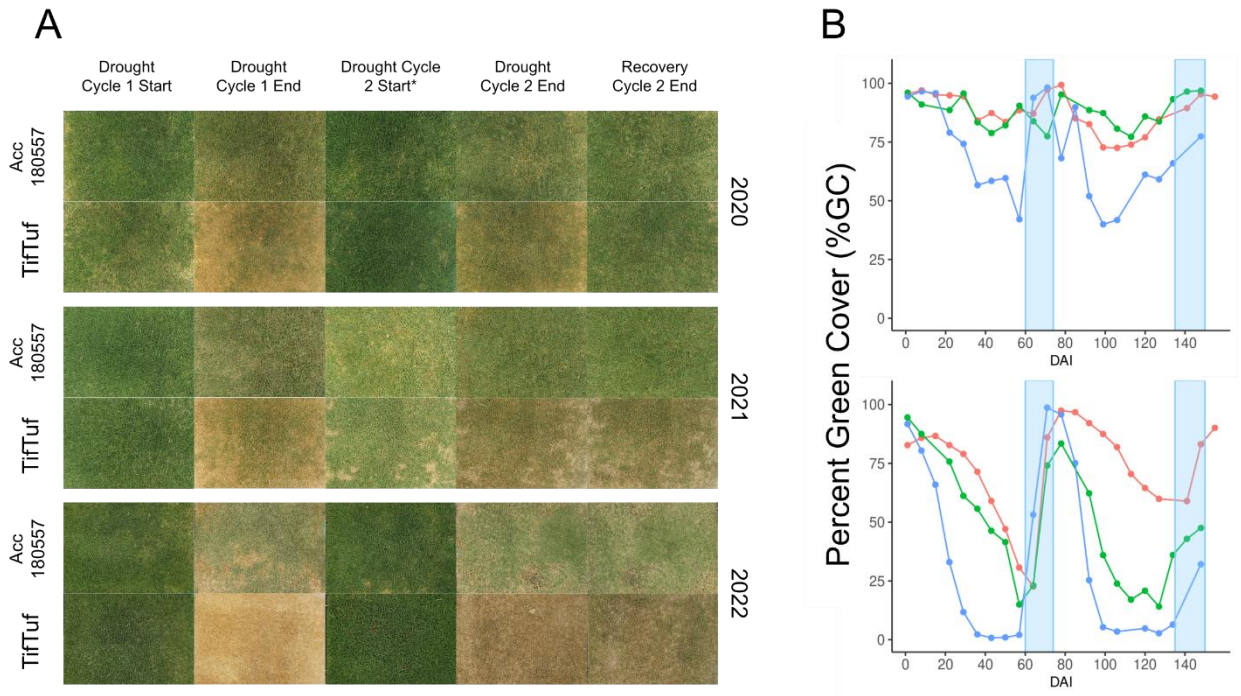

Figure 2.6 Comparison of drought response between TifTuf and Acc180557. (A) Images were taken at the starts and ends of each dry-down cycle and recovery period for 2020, 2021, and 2022. The images for the column “Drought Cycle 2 Start” correspond with the “Recovery Cycle 1 End” date. (B) Line graphs comparing the mean percent green cover over the number of days after initiation (DAI) between Acc180557 (top) and TifTuf (bottom) for 2020 (red lines), 2021 (green lines), and 2022 (blue lines). Blue shaded regions represent the recovery periods (14-days) between each dry-down cycle.

Conclusion

In summary, this study demonstrates a range of reactions of a large set of bermudagrass accessions to multiple drought and recovery periods during annual long-term periods of drought. Differences in drought responses were apparent among the accessions, suggesting that there may exist a sizable amount of genetic variation among tested accessions that is promising for future breeding efforts. The study also observed differences in both drought responses and recovery rates between years and between

dry-down cycles and is the first known study to point to potential stress memory as viewed from the turfgrass breeding standpoint. Widely grown cultivars responded moderately well to drought stress but were not among the top performers in this study. Information from this project should be used to improve methodologies for selecting for drought tolerance in turfgrass breeding programs and be used to incentivize the switch to warm-season grasses like bermudagrass to reduce water inputs.

References

Baird, J.H., Lukaszewski, A.J., Pudzianowska, M., Bowman, C.S. (2023) US Patent for Bermudagrass named 'UCR 17-8' (U.S. Patent No. PP 35,357). https://patents.justia.com/patent/PP35357

Baird, J.H., Lukaszewski, A.J., Pudzianowska, M., Bowman, C.S. (2023) US Patent for Bermudagrass named 'UCR TP6-3' (U.S. Patent No. PP 35,441). https://patents.justia.com/patent/PP35441

Baldwin, C. M., Liu, H., McCarty, L. B., Bauerle, W. L., & Toler, J. E. (2006). Response of Six Bermudagrass Cultivars to Different Irrigation Intervals. HortTechnology, 16(3), 466–470. https://doi.org/10.21273/HORTTECH.16.3.0466

Bi, A., Wang, T., Wang, G., Zhang, L., Wassie, M., Amee, M., Xu, H., Hu, Z., Liu, A., Fu, J., Chen, L., & Hu, T. (2021). Stress memory gene FaHSP17.8-CII controls thermotolerance via remodeling PSII and ROS signaling in tall fescue. Plant Physiology, 187(3), 1163–1176. https://doi.org/10.1093/plphys/kiab205

Burton, G. W. (1981). Tifway II bermudagrass released [Turf, mutants]. USGA Green Section Record, 19.

Carrow, R. N. (1995). Drought Resistance Aspects of Turfgrasses in the Southeast: Evapotranspiration and Crop Coefficients. Crop Science, 35(6), 1685–1690. https://doi.org/10.2135/cropsci1995.0011183X003500060029x

Carrow, R. N. (1996). Drought Resistance Aspects of Turfgrasses in the Southeast: Root-Shoot Responses. Crop Science, 36(3), cropsci1996.0011183X003600030028x. https://doi.org/10.2135/cropsci1996.0011183X003600030028x

Close, T. J. (1997). Dehydrins: A commonalty in the response of plants to dehydration and low temperature. Physiologia Plantarum, 100(2), 291–296. https://doi.org/10.1111/j.1399-3054.1997.tb04785.x

Ding, Y., Fromm, M., & Avramova, Z. (2012). Multiple exposures to drought "train" transcriptional responses in Arabidopsis. Nature Communications, 3(1), Article 1. https://doi.org/10.1038/ncomms1732

Ding, Y., Liu, N., Virlouvet, L., Riethoven, J.-J., Fromm, M., & Avramova, Z. (2013). Four distinct types of dehydration stress memory genes in Arabidopsis thaliana. BMC Plant Biology, 13(1), Article 1. https://doi.org/10.1186/1471-2229-13-229

Fan, J., Zhang, W., Amombo, E., Hu, L., Kjorven, J. O., & Chen, L. (2020). Mechanisms of Environmental Stress Tolerance in Turfgrass. Agronomy, 10(4), Article 4. https://doi.org/10.3390/agronomy10040522

Fang, Y., & Xiong, L. (2015). General mechanisms of drought response and their application in drought resistance improvement in plants. Cellular and Molecular Life Sciences, 72(4), 673–689. https://doi.org/10.1007/s00018-014-1767-0

Hejl, R. W., Wherley, B. G., White, R. H., Thomas, J. C., & Fontanier, C. H. (2016). Deficit Irrigation and Simulated Traffic on 'Tifway' Bermudagrass Summer Performance and Autumn Recovery. Crop Science, 56(2), 809–817. https://doi.org/10.2135/cropsci2015.03.0197

Herzig, P., Borrmann, P., Knauer, U., Klück, H.-C., Kilias, D., Seiffert, U., Pillen, K., & Maurer, A. (2021). Evaluation of RGB and Multispectral Unmanned Aerial Vehicle (UAV) Imagery for High-Throughput Phenotyping and Yield Prediction in Barley Breeding. Remote Sensing, 13(14), Article 14. https://doi.org/10.3390/rs13142670

Hu, L., Wang, Z., Du, H., & Huang, B. (2010). Differential accumulation of dehydrins in response to water stress for hybrid and common bermudagrass genotypes differing in drought tolerance. Journal of Plant Physiology, 167(2), 103–109. https://doi.org/10.1016/j.jplph.2009.07.008

Hu, L., Wang, Z., & Huang, B. (2009). Photosynthetic Responses of Bermudagrass to Drought Stress Associated with Stomatal and Metabolic Limitations. Crop Science, 49(5), 1902–1909. https://doi.org/10.2135/cropsci2008.12.0697

Hu, T., Jin, Y., Li, H., Amombo, E., & Fu, J. (2016). Stress memory induced transcriptional and metabolic changes of perennial ryegrass (Lolium perenne) in response to salt stress. Physiologia Plantarum, 156(1), 54–69. https://doi.org/10.1111/ppl.12342

Hu, T., Liu, S.-Q., Amombo, E., & Fu, J.-M. (2015). Stress memory induced rearrangements of HSP transcription, photosystem II photochemistry and metabolism of tall fescue (Festuca arundinacea Schreb.) in response to high-temperature stress. Frontiers in Plant Science, 6. https://www.frontiersin.org/articles/10.3389/fpls.2015.00403

Huang, B. (2008). Turfgrass water requirements and factors affecting water usage. Water Quality and Quantity Issues for Turfgrass in Urban Landscapes. CAST Spec. Publ, 27, 193–205.

Huang, B., & Fry, J. D. (2000). Turfgrass Evapotranspiration. Journal of Crop Production, 2(2), 317–333. https://doi.org/10.1300/J144v02n02_14

Jacques, C., Salon, C., Barnard, R. L., Vernoud, V., & Prudent, M. (2021). Drought Stress Memory at the Plant Cycle Level: A Review. Plants, 10(9), Article 9. https://doi.org/10.3390/plants10091873

Karcher, D. E., Purcell, C. J., Richardson, M. D., Purcell, L. C., & Hignight, K. W. (2017, October 24). A New Java Program to Rapidly Quantify Several Turfgrass Parameters from Digital Images. ASA, CSSA and SSSA International Annual (2017). https://scisoc.confex.com/scisoc/2017am/meetingapp.cgi/Paper/109313

Karcher, D. E., & Richardson, M. D. (2013). Digital Image Analysis in Turfgrass Research. In Turfgrass: Biology, Use, and Management (pp. 1133-1149–2). John Wiley & Sons, Ltd. https://doi.org/10.2134/agronmonogr56.c29

Karcher, D. E., Richardson, M. D., Hignight, K., & Rush, D. (2008). Drought Tolerance of Tall Fescue Populations Selected for High Root/Shoot Ratios and Summer Survival. Crop Science, 48(2), 771–777. https://doi.org/10.2135/cropsci2007.05.0272

Katuwal, K. B., Jespersen, D., Bhattarai, U., Chandra, A., Kenworthy, K. E., Milla-Lewis, S. R., Schwartz, B. M., Wu, Y., & Raymer, P. (2022). Multilocational screening identifies new drought-tolerant, warm-season turfgrasses. Crop Science, 62(4), 1614–1630. https://doi.org/10.1002/csc2.20726

Katuwal, K. B., Schwartz, B., & Jespersen, D. (2020). Desiccation avoidance and drought tolerance strategies in bermudagrasses. Environmental and Experimental Botany, 171, 103947. https://doi.org/10.1016/j.envexpbot.2019.103947

Kenna, M. P. (2008). Turfgrass and the environment. Water Quality and Quantity Issues for Turfgrasses in Urban Landscapes. CAST Spec. Publ, 27, 65–90.

Levitt, J. (1980). Responses of Plants to Environmental Stress, 2nd Edition, Volume 1: Chilling, Freezing, and High Temperature Stresses. Responses of Plants to Environmental Stress, 2nd Edition, Volume 1: Chilling, Freezing, and High Temperature Stresses. https://www.cabdirect.org/cabdirect/abstract/19802605739

Lobell, D. B., & Gourdji, S. M. (2012). The Influence of Climate Change on Global Crop Productivity. Plant Physiology, 160(4), 1686–1697. https://doi.org/10.1104/pp.112.208298

Lu, S., Chen, C., Wang, Z., Guo, Z., & Li, H. (2009). Physiological responses of somaclonal variants of triploid bermudagrass (Cynodon transvaalensis × Cynodon dactylon) to drought stress. Plant Cell Reports, 28(3), 517–526. https://doi.org/10.1007/s00299-008-0649-z

Minor, J., Campbell, B., Waltz, C., & Berning, J. (2020). Water Savings and Return on Investment of a New Drought Resistant Turfgrass. Journal of Environmental Horticulture, 38(2), 56–62. https://doi.org/10.24266/0738-2898-38.2.56

Morris, K. N., & Shearman, R. C. (1998). NTEP turfgrass evaluation guidelines. NTEP Turfgrass Evaluation Workshop, Beltsville, MD, 1–5.

Onofri, A. (2020). Aomisc: Statistical methods for the agricultural sciences. R Package Version 0.62.

Pierce, O. S., Francis J. (2001). Contemporary Statistical Models for the Plant and Soil Sciences. CRC Press. https://doi.org/10.1201/9781420040197

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., Heisterkamp, S., Van Willigen, B., & Maintainer, R. (2017). Package 'nlme.' Linear and Nonlinear Mixed Effects Models, Version, 3(1), 336.

Qian, Y. L., Fry, J. D., & Upham, W. S. (1997). Rooting and Drought Avoidance of Warm-Season Turfgrasses and Tall Fescue in Kansas. Crop Science, 37(3), cropsci1997.0011183X003700030034x. https://doi.org/10.2135/cropsci1997.0011183X003700030034x

Riley, R. J. (2000). Cynodon dactylon plant named `Riley's Super Sport` (United States Patent USPP11181P). https://patents.google.com/patent/USPP11181P/en

Ritz, C., Strebig, J. C., & Ritz, M. C. (2016). Package 'drc.' Creative Commons: Mountain View, CA, USA.

Robins, J. G., & Bushman, B. S. (2020). Turfgrass performance of perennial wheatgrass species when grown as monocultures and mixtures. Agronomy Journal, 112(5), 3567–3578. https://doi.org/10.1002/agj2.20346

RStudio Team. (2020). RStudio: Integrated Development Environment for R. RStudio, PBC. http://www.rstudio.com/

Saville, D. J. (2015). Multiple Comparison Procedures—Cutting the Gordian Knot. Agronomy Journal, 107(2), 730–735. https://doi.org/10.2134/agronj2012.0394

Schwartz, B. M., Hanna, W. W., Baxter, L. L., Raymer, P. L., Waltz, F. C., Kowalewski, A. R., Chandra, A., Genovesi, A. D., Wherley, B. G., Miller, G. L., Milla-Lewis, S. R., Reynolds, W. C., Wu, Y., Martin, D. L., Moss, J. Q., Kenna, M. P., Unruh, J. B., Kenworthy, K. E., Zhang, J., & Munoz, P. R. (2018). 'DT-1', a Drought-tolerant Triploid Turf Bermudagrass. HortScience, 53(11), 1711–1714. https://doi.org/10.21273/HORTSCI13083-18

Severmutlu, S., Mutlu, N., Gurbuz, E., Gulsen, O., Hocagil, M., Karaguzel, O., Heng-Moss, T., Shearman, R. C., & Gaussoin, R. E. (2011). Drought Resistance of Warm-season Turfgrasses Grown in Mediterranean Region of Turkey. HortTechnology, 21(6), 726–736. https://doi.org/10.21273/HORTTECH.21.6.726

Shi, H., Wang, Y., Cheng, Z., Ye, T., & Chan, Z. (2012). Analysis of Natural Variation in Bermudagrass (Cynodon dactylon) Reveals Physiological Responses Underlying Drought Tolerance. PLOS ONE, 7(12), e53422. https://doi.org/10.1371/journal.pone.0053422

Spiess, A.-N., & Neumeyer, N. (2010). An evaluation of R2 as an inadequate measure for nonlinear models in pharmacological and biochemical research: A Monte Carlo approach. BMC Pharmacology, 10(1), Article 1. https://doi.org/10.1186/1471-2210-10-6

Steinke, K., Chalmers, D., Thomas, J., & White, R. (2011). Bermudagrass and Buffalograss Drought Response and Recovery at Two Soil Depths. Crop Science, 51(3), 1215–1223. https://doi.org/10.2135/cropsci2010.08.0469

Su, K., Moss, J. Q., Zhang, G., Martin, D. L., & Wu, Y. (2013). Bermudagrass Drought Tolerance Associated with Dehydrin Protein Expression during Drought Stress. Journal of the American Society for Horticultural Science, 138(4), 277–282. https://doi.org/10.21273/JASHS.138.4.277

Thapa, S. B. (2011). Evaluation of twenty bermudagrass cultivars for their drought resistance [M.S., Oklahoma State University]. In ProQuest Dissertations and Theses (877985297). ProQuest Dissertations & Theses A&I; ProQuest Dissertations & Theses Global. https://www.proquest.com/dissertations-theses/evaluation-twenty-bermudagrass-cultivars-their/docview/877985297/se-2?accountid=14521

Walter, J., Nagy, L., Hein, R., Rascher, U., Beierkuhnlein, C., Willner, E., & Jentsch, A. (2011). Do plants remember drought? Hints towards a drought-memory in grasses. Environmental and Experimental Botany, 71(1), 34–40. https://doi.org/10.1016/j.envexpbot.2010.10.020

Yang, G., Liu, J., Zhao, C., Li, Z., Huang, Y., Yu, H., Xu, B., Yang, X., Zhu, D., Zhang, X., Zhang, R., Feng, H., Zhao, X., Li, Z., Li, H., & Yang, H. (2017). Unmanned Aerial Vehicle Remote Sensing for Field-Based Crop Phenotyping: Current Status and Perspectives. Frontiers in Plant Science, 8. https://www.frontiersin.org/articles/10.3389/fpls.2017.01111

Zhang, J., Poudel, B., Kenworthy, K., Unruh, J. B., Rowland, D., Erickson, J. E., & Kruse, J. (2019). Drought responses of above-ground and below-ground characteristics in warm-season turfgrass. Journal of Agronomy and Crop Science, 205(1), 1–12. https://doi.org/10.1111/jac.12301

Zhou, P., An, Y., Wang, Z., Du, H., & Huang, B. (2014). Characterization of Gene Expression Associated with Drought Avoidance and Tolerance Traits in a Perennial Grass Species. PLOS ONE, 9(8), e103611. https://doi.org/10.1371/journal.pone.0103611

Zhou, Y., Lambrides, C. J., & Fukai, S. (2013). Drought Resistance of C4 Grasses Under Field Conditions: Genetic Variation Among a Large Number of Bermudagrass (Cynodon spp.) Ecotypes Collected from Different Climatic Zones. Journal of Agronomy and Crop Science, 199(4), 253–263. https://doi.org/10.1111/jac.12020

Zhou, Y., Lambrides, C., Kearns, R., Ye, C., Cao, N., & Fukai, S. (2009). Selecting for drought tolerance among Australian green couch grasses (Cynodon spp.). Crop and Pasture Science, 60(12), 1175. https://doi.org/10.1071/CP09172

Supplemental Tables

Supplemental tables S1-5 are similar to Table 2, but show a comparison of mean performance values between all accessions for percent green cover (Table S1), NDVI (Table S2), visual quality (Table S3), leaf firing (Table S4), and DGCI (Table S5) using Fisher's LSD.

Supplemental table S6 describes the stress memory "type" of each accession as measured by the percent change of the slope for the nonlinear regression models between Cycle 1 and Cycle 2.

Supplemental tables can be made available upon request.

Supplemental Table S2.1 Comparison of percent green coverage (%GC) at five time points each year for 70 bermudagrass entries using Fisher's Protected Least Significant Difference test. Means not sharing a letter are significantly different at the 5% level of significance according to a F-test.

Supplemental Table S2.2 Comparison of normalized difference vegetation index (NDVI) at five time points each year for 70 bermudagrass entries using Fisher's Protected Least Significant Difference test. Means not sharing a letter are significantly different at the 5% level of significance according to a F-test.

Supplemental Table S2.3 Comparison of visual quality (VQ) at five time points each year for 70 bermudagrass entries using Fisher's Protected Least Significant Difference test. Means not sharing a letter are significantly different at the 5% level of significance according to a F-test.

Supplemental Table S2.4 Comparison of leaf firing (LF) at five time points each year for 70 bermudagrass entries using Fisher's Protected Least Significant Difference test. Means not sharing a letter are significantly different at the 5% level of significance according to a F-test.

Supplemental Table S2.5 Comparison of dark green color index (DGCI) at five time points each year for 70 bermudagrass entries using Fisher's Protected Least Significant Difference test. Means not sharing a letter are significantly different at the 5% level of significance according to a F-test.

Supplemental Table S2.6 List of bermudagrass genotypes and their stress memory type.

Chapter 3

Exploration of DNA marker associations with winter color retention in bermudagrass (*Cynodon* spp.)

Abstract

Winter color retention is a major target for improvement of bermudagrass in the southwestern United States. As climates shift towards hotter and drier, it becomes increasingly urgent to adopt warm-season grasses to such conditions, including bermudagrass (*Cynodon* spp.). This is especially significant in the context of reducing the irrigation inputs while maintaining plant health and visual appea. The goal of this study was to evaluate winter color retention among a population of inter- and intraspecific hybrids of bermudagrass and to attempt to associate DNA polymorphism data with phenotypic values. A total of 179 accessions, including six commercial standards, were evaluated in four separate studies across a minimum of two years. The set of accessions was highly heterogenous, including ploidy level differences, and attempts were made to correct for these through statistical methods. Highly significant associations between DNA markers and phenotyping values were detected. However, given the heterogeneity of the tested set, additional validation studies are necessary. The study is a clear step forward in bermudagrass genomics, though complicated by the lack of standard genomic resources available in other crops.

Introduction

Turfgrasses are functional crops that serve a variety of roles and hold a substantial recreational, environmental, and economic importance (Breuninger et al., 2013; Casler & Duncan, 2003; Stier et al., 2013). One of the major features of turfgrass is its aesthetics, with much emphasis placed on visual appeal (Ghimire et al., 2019; Yue et al., 2017). Turfgrasses can be divided into two types, cool-season or warm-season, each having its own benefits and drawbacks. Cool-season grasses are typically able to maintain green color all year but have much higher irrigation requirements than warm-season grasses (Huang 2008; Huang and Fry 2000; Kenna 2008). Warm-season grasses, on the other hand, are well adapted to hot and dry conditions and require less water overall (Huang and Fry 2000), but are unable to maintain green color at lower temperatures and enter a winter dormancy stage. With global climates shifting towards hotter and drier conditions, there is an increasing demand for warm-season grasses, such as bermudagrass (*Cynodon* spp.), that can retain their green color all year.

Bermudagrasses, like most warm-season grasses, are not well adapted to colder conditions but variation in responses to low temperatures is evident. Although they are naturally adapted to warmer tropical and subtropical climates, bermudagrass has been found in colder climates outside of its natural distribution range and has been collected as far north as 53°N latitude (Hanna, Raymer, and Schwartz 2013; Harlan, de Wet, and Rawal 1970; Taliaferro 2003). In the United States, Bermudagrass can be found throughout the transition zone, though its

susceptibility to stress and winterkill at lower temperatures hinder its widespread use in colder climates (Taliaferro, Rouquette Jr., and Mislevy 2004). The transition zone is a climate zone that extends through the central part of the United States where both cool- and warm-season grasses can grow, though neither are fully adapted. Here, low temperatures during the winter can injure warm-season grasses such as bermudagrass, while high temperatures during the summer can facilitate ideal growing conditions. At low temperatures (LT) between 0–15°C, bermuda turf experiences chilling stress and tends to decline in quality due to the inhibition of physiological and metabolic processes, such as photosynthesis and respiration (Bertrand et al. 2013; Levitt 1980). Freezing stress below 0°C damages cell membranes due to the formation of ice crystals (Goswami et al. 2022). Dormancy onset typically occurs around 10°C and can be visually characterized in bermudagrass by reduced growth and degradation of chlorophyll within leaf cells, turning them to straw-brown color. This is highly unappealing to most end users. Breeding programs have made an effort to generate and select interspecific hybrids between *Cynodon transvaalensis* x *Cynodon dactylon* for improved cold tolerance traits, such as winter color retention (WCR) and/or spring green-up (SGU). A recent cultivar, ‘Tahoma 31’, has shown promising results in regards to improved winter survival and spring green-up, as it was developed in the transition zone (Fontanier et al. 2020; Gopinath, Moss, and Wu 2021).

A recent study has shown improved winter hardiness among *C. transvaalensis* accessions (Yu et al. 2022). These accessions showed reduced dormancy periods, resulting in faster spring green-up. Winter survival in bermudagrass differs from winter color retention. Although similar, these two types of responses have different physiological mechanisms and different meanings to turfgrass breeders and consumers. Improved winter hardiness and survivability are highly dependent on acclimation to colder temperatures which improves freezing tolerance. One of the objectives for improving winter survivability, especially in regions such as the transition zone throughout the United States, is to also select for earlier spring green-up. This implies a reduced dormancy period and, therefore, improved tolerance to the cold (Bertrand et al. 2013). Winter color retention, however, is a trait that is more important to southern and southwestern regions of the United States where non-dormant turf is desired year-round. Improved winter color retention is defined by the retention of green color throughout the winter months, avoiding the natural dormancy period of bermudagrass at colder temperatures. At the University of California, Riverside, selection for improved winter color retention is one of the major objectives for bermudagrass breeding.

Responses to lower temperatures have been characterized in bermudagrass at the physiological and biochemical level in both controlled environments and field studies. Some morphological traits, such as stolon and rhizome sizes, have been found to be positively correlated with cold tolerance,

while some others, such as leaf texture (where finer textures are more susceptible to the cold), have been found to be negatively correlated (Ahring et al. 1975; Stefaniak et al. 2009). These findings show that bermudagrasses with more robust features are typically more winter hardy. At the biochemical level, several stress-response metabolites including proline, dehydrins, abscisic acid (ABA), and anthocyanins have been reported to increase in response to the chilling stress induced by cold acclimation (Zhang, Ervin, et al. 2011; Zhang, Wang, et al. 2011). Morphological features and biochemical pathways can also change in parallel. Reduced day length and temperatures were found to increase rooting depth and root fresh weight, but only reduced temperatures were found to increase the accumulation of certain sugars in roots and shoots (Esmaili and Salehi 2012). Additionally, Esmaili and Salehi (2012) proposed that by increasing the daylength during winter months may allow bermudagrass to overcome, or display lower severity of, dormancy-related responses under lower temperatures.

Management practices have also been studied in an attempt to provide field managers with a solution to reducing the effects of cold stress. Proper fertilization, mowing, and irrigation schedules can enhance winter survivability and reduce the potential for injury or disease. Chemical applications have also been studied in the context of alleviating cold stress for bermudagrasses. The exogenous application of melatonin, for example, has been shown to improve cell membrane stability, photosynthetic performance, and the activity of antioxidant enzymes under cold stress (Hu et al. 2016). Antioxidant enzymes,

such as superoxide dismutase (SOD), peroxidase (POD), and ascorbate peroxidase (APX), protect plant cells from damage by reactive oxygen species (ROS) that accumulate in response to oxidative stress. Cold stress can induce oxidative stress, leading to increased activity of these enzymes. The exogenous application of ABA has also been studied, in which the application of ABA to bermudagrass plants under chilling stress was able to enhance cold tolerance by upregulating the expression of stress-defensive genes involved in the ABA-dependent pathway (Cheng et al. 2016; Huang et al. 2017).

In the Southwest of the United States, enhancing winter color retention is a major objective for bermudagrass breeding efforts. In this study, a mixed population of 179 bermudagrass accessions from multiple field trials was evaluated for their winter color retention during the winter months at the University of California, Riverside (UCR). A genome-wide association study (GWAS) was performed on the population in an attempt to identify genetic markers associated with winter color retention. Such markers would potentially provide breeders with new tools to screen bermudagrass hybrids for winter color retention through marker-assisted selection. As genomic tools in bermudagrass are sparce, this study attempted to open new grounds by exploring a range of approaches never tested in this crop.

Methods

Plant materials

A total of 179 accessions were selected for this study. Of those, 26 were from the UCR bermudagrass germplasm collection, 147 were from the 2018 UCR bermudagrass nursery, and six were commercial standards (‘Bandera’, ‘Coachella’, ‘Presidio’, ‘Santa Ana’, ‘Tahoma 31’, ‘TifTuf’). Nine accessions were randomly selected for an additional replicate for internal validation (Table 3.1). Accessions from both the germplasm collection and the nursery were randomly selected to cover the range of phenotypic responses observed in these populations.

The UCR bermudagrass germplasm collection was established with plant material obtained from the United States Department of Agriculture– Agricultural Research Service National Plant Germplasm System (USDA-ARS NPGS) as well as that donated by Dr. Jeff Krans (Professor Emeritus, Mississippi State University) as previously described (Pudzianowska and Baird 2021). The 2018 UCR bermudagrass nursery was established using progeny derived from open-pollination of accessions in the germplasm collection. A total of 770 nursery accessions were grown from seed under greenhouse conditions, and planted

Table 3.1 List of bermudagrass accessions and their study origin used in a genome-wide association study. Accessions originated from the germplasm collection (A), 2018 nursery (B), 2017 trial (C), and 2019 NTEP (D) at the University of California, Riverside.

Accession	Entry type	Source	Accession	Entry type	Source	Accession	Entry type	Source
Bandera	Standard Entry	C	B162	Germplasm	A	UCRC180109	Hybrid	B
Coachella	Standard Entry	C, D	B163	Germplasm	A	UCRC180114	Hybrid	B
Presidio	Standard Entry	C, D	UCRC180009	Hybrid	B	UCRC180115	Hybrid	B
Santa Ana	Standard Entry	C	UCRC180010	Hybrid	B	UCRC180120	Hybrid	B
Tahoma 31[a]	Standard Entry	C, D	UCRC180012[a]	Hybrid	B	UCRC180127	Hybrid	B
TifTuf	Standard Entry	C, D	UCRC180014	Hybrid	B	UCRC180129	Hybrid	B
B002	Germplasm	A	UCRC180017	Hybrid	B	UCRC180136	Hybrid	B
B005	Germplasm	A	UCRC180018	Hybrid	B	UCRC180139	Hybrid	B
B037	Germplasm	A	UCRC180021	Hybrid	B	UCRC180140	Hybrid	B
B038	Germplasm	A	UCRC180024	Hybrid	B	UCRC180143	Hybrid	B
B043	Germplasm	A	UCRC180039	Hybrid	B	UCRC180145	Hybrid	B
B044	Germplasm	A	UCRC180044	Hybrid	B	UCRC180146[a]	Hybrid	B
B056	Germplasm	A	UCRC180049	Hybrid	B	UCRC180149	Hybrid	B
B068A	Germplasm	A	UCRC180055	Hybrid	B	UCRC180151	Hybrid	B
B094	Germplasm	A	UCRC180060	Hybrid	B	UCRC180164	Hybrid	B
B096	Germplasm	A	UCRC180063	Hybrid	B	UCRC180169	Hybrid	B
B101	Germplasm	A	UCRC180066	Hybrid	B	UCRC180170	Hybrid	B
B102	Germplasm	A	UCRC180068	Hybrid	B	UCRC180174	Hybrid	B
B105	Germplasm	A	UCRC180070	Hybrid	B	UCRC180175[a]	Hybrid	B
B106	Germplasm	A	UCRC180075	Hybrid	B	UCRC180176	Hybrid	B
B110	Germplasm	A	UCRC180077	Hybrid	B	UCRC180177	Hybrid	B
B116	Germplasm	A	UCRC180078	Hybrid	B	UCRC180193	Hybrid	B
B120	Germplasm	A	UCRC180080	Hybrid	B	UCRC180198	Hybrid	B
B121	Germplasm	A	UCRC180084	Hybrid	B	UCRC180199	Hybrid	B
B129[a]	Germplasm	A	UCRC180090	Hybrid	B	UCRC180207	Hybrid	B
B131	Germplasm	A	UCRC180094	Hybrid	B	UCRC180221	Hybrid	B
B132	Germplasm	A	UCRC180095	Hybrid	B	UCRC180225	Hybrid	B
B137	Germplasm	A	UCRC180099	Hybrid	B	UCRC180240	Hybrid	B
B144	Germplasm	A	UCRC180100	Hybrid	B	UCRC180241[a]	Hybrid	B
B159	Germplasm	A	UCRC180104	Hybrid	B	UCRC180247	Hybrid	B

[a] Replicated for internal validation.

Table 3.1 (Continued)

Accession	Entry type	Source	Accession	Entry type	Source	Accession	Entry type	Source
UCRC180282	Hybrid	B	UCRC180522	Hybrid	B	UCRC180633	Hybrid	B
UCRC180296	Hybrid	B	UCRC180524	Hybrid	B	UCRC180636	Hybrid	B
UCRC180319	Hybrid	B	UCRC180526	Hybrid	B	UCRC180637	Hybrid	B
UCRC180335	Hybrid	B	UCRC180528	Hybrid	B	UCRC180643	Hybrid	B
UCRC180348	Hybrid	B	UCRC180530	Hybrid	B	UCRC180653	Hybrid	B
UCRC180351	Hybrid	B	UCRC180532	Hybrid	B	UCRC180697	Hybrid	B
UCRC180352	Hybrid	B	UCRC180540	Hybrid	B	UCRC180703	Hybrid	B
UCRC180374	Hybrid	B	UCRC180549	Hybrid	B	UCRC180704	Hybrid	B
UCRC180387	Hybrid	B	UCRC180557	Hybrid	B	UCRC180707	Hybrid	B
UCRC180390	Hybrid	B	UCRC180559	Hybrid	B	UCRC180708	Hybrid	B
UCRC180402	Hybrid	B	UCRC180566	Hybrid	B	UCRC180713	Hybrid	B
UCRC180405	Hybrid	B	UCRC180572	Hybrid	B	UCRC180714	Hybrid	B
UCRC180410	Hybrid	B	UCRC180576[a]	Hybrid	B	UCRC180719	Hybrid	B
UCRC180414	Hybrid	B	UCRC180578	Hybrid	B	UCRC180725	Hybrid	B
UCRC180420	Hybrid	B	UCRC180579	Hybrid	B	UCRC180726	Hybrid	B
UCRC180421	Hybrid	B	UCRC180581	Hybrid	B	UCRC180735	Hybrid	B
UCRC180430[a]	Hybrid	B	UCRC180582	Hybrid	B	UCRC180739	Hybrid	B
UCRC180431	Hybrid	B	UCRC180588	Hybrid	B	UCRC180740	Hybrid	B
UCRC180441	Hybrid	B	UCRC180591	Hybrid	B	UCRC180743	Hybrid	B
UCRC180442	Hybrid	B	UCRC180593	Hybrid	B	UCRC180747	Hybrid	B
UCRC180448	Hybrid	B	UCRC180594	Hybrid	B	UCRC180748	Hybrid	B
UCRC180451	Hybrid	B	UCRC180595	Hybrid	B	UCRC180750	Hybrid	B
UCRC180458	Hybrid	B	UCRC180611	Hybrid	B	UCRC180753	Hybrid	B
UCRC180472	Hybrid	B	UCRC180613	Hybrid	B	UCRC180756	Hybrid	B
UCRC180489	Hybrid	B	UCRC180615	Hybrid	B	UCRC180761	Hybrid	B
UCRC180490	Hybrid	B	UCRC180616	Hybrid	B	UCRC180763	Hybrid	B
UCRC180498	Hybrid	B	UCRC180624	Hybrid	B	UCRC180766	Hybrid	B
UCRC180504	Hybrid	B	UCRC180626	Hybrid	B	UCRC180768	Hybrid	B
UCRC180520	Hybrid	B	UCRC180627	Hybrid	B	UCRC180769[a]	Hybrid	B
UCRC180521	Hybrid	B	UCRC180631	Hybrid	B			

[a] Replicated for internal validation.

in the field at UCR Experiment Station in June 2018. Individual plants (accessions) were grown in the field in plots of 0.61 m x 0.61 m dimensions in Buren fine sandy loam soil as an unreplicated trial. Five of the commercial standards (‘Bandera’, ‘Coachella’, ‘Presidio’, ‘Santa Ana’, ‘TifTuf’) were established in May 2017 as part of a separate trial. ‘Tahoma 31’ was present in the 2019 National Bermudagrass Test from the National Turfgrass Evaluation Program (NTEP) with ‘Coachella’, ‘Presidio’, and ‘TifTuf’ and was established in June 2019. The 2019 NTEP test was established to evaluate advanced breeding lines from several bermudagrass breeding programs across the United States in multiple locations. Tested accessions originated from various crosses with unknown male parent, and from collection accessions. Many are, or are assumed to be, interspecific hybrids. As explained clearly in Chapter 1, tested accessions also varied in ploidy levels, which included diploids, triploids and tetraploids. Ploidy differences complicated data analyses and required specialized statistical tools.

Winter color retention and turf quality

Field grown plants were not subjected to any experimental treatment and were visually evaluated for their performance under standard maintenance practices. Visual assessment was based on a 1 to 9 scale, with quality (VQ) scored of 1 indicated being poor or dead turf and 9 being ideal turf, while color (VC) with the score of 1 being straw brown and 9 being dark green (Morris and

Shearman 1998). These ratings were recorded during the winter months (December, January, February) from January 2021 to January 2022 and January 2019 to February 2021 for the germplasm collection and nursery, respectively. For the 2017 trial, plants were grown in the field on plots 1.52 m x 1.52 m dimensions in Buren fine sandy loam soil in three replicates following a randomized complete block design (RCBD). Plants were not subjected to any experimental treatment until April 2018, whereafter data was not included in this study. Accessions for the 2019 NTEP test were grown on plots 1.52 m x 1.52 m in Handford coarse sandy loam in three replicates following a RCBD. Plants were initially subjected to reduced irrigation at 40% weekly reference evapotranspiration (ET_o) replacement, but were quickly switched to 35% ET_o, from August 2020 to October 2020 and from July 2021 to October 2021. Accessions underwent visual evaluation of VQ and VC during the winter months from December 2017 to February 2018 and December 2019 to February 2022 for the 2017 trial and 2019 NTEP test, respectively. Monthly weather data such as average evapotranspiration, precipitation, and high and low temperatures were recorded by a California Irrigation Management Information System (CIMIS) weather station (U.C. Riverside #44) located near the field trials at the University of California, Riverside (Table 3.2). Plot fertilization schedules for all studies are listed in Supplementary Table S3.1.

Table 3.2 Monthly weather data collected in 2018 to 2022 from the California Irrigation Management Information System (CIMIS) weather station (#44) at the University of California, Riverside.

	Total		Average								
Month	ETo mm	Precip mm	Sol Rad W/m^2	Vap Pres kPa	Max Air Temp °C	Min Air Temp °C	Air Temp °C	Max Rel Hum %	Min Rel Hum %	Rel Hum %	Soil Temp °C
Jan-2018	61.16	42	111	0.9	22.6	8.5	14.8	78	31	52	12.8
Feb-2018	80.44	7.6	166	0.7	21	6.5	13.4	76	24	47	13.5
Mar-2018	96.89	41.6	181	1.0	20.7	8.7	14.4	88	39	62	15.7
Apr-2018	144.63	0.0	246	1.0	24.9	10.9	17.4	82	32	54	18.4
May-2018	141.42	6.8	268	1.3	24.1	12.6	17.6	85	45	64	20.4
Jun-2018	193.27	0.0	353	1.5	30.2	14.9	21.7	85	34	58	23.3
Jul-2018	204.21	1.0	315	1.8	35.4	19.8	27.1	78	31	50	26.0
Aug-2018	186.74	0.0	291	1.8	33.8	19.1	25.6	81	32	54	25.5
Sep-2018	148.87	0.1	251	1.6	32.3	16.0	23.1	84	31	56	23.2
Oct-2018	109.21	24.5	196	1.2	26.8	13.7	19.7	79	33	55	20.1
Nov-2018	79.54	21.3	153	0.8	23.6	9.7	16.3	66	26	44	15.2
Dec-2018	56.91	25.6	123	0.8	19.3	7.3	12.5	81	33	56	12.3
Jan-2019	58.22	65.7	127	0.8	18.9	7.7	12.9	77	38	55	11.3
Feb-2019	60.27	117.6	156	0.8	16.3	5.7	10.7	82	40	61	11.1
Mar-2019	110.76	37.0	214	1.0	21.4	9.7	15.2	76	36	56	14.7
Apr-2019	149.83	1.5	264	1.1	25.3	12.3	18.2	78	33	53	18.2
May-2019	125.82	24.6	253	1.3	21.8	11.5	16.0	91	50	70	19.4
Jun-2019	164.8	0.5	308	1.6	28.4	15.6	21.0	87	42	64	23.0
Jul-2019	203.98	0.2	337	1.6	32.9	17.0	24.3	81	32	53	23.9
Aug-2019	195.16	0.0	314	1.5	34.1	16.9	24.8	81	25	49	23.5
Sep-2019	146.39	0.2	248	1.4	30.9	16.7	23.2	76	31	52	23.6
Oct-2019	129.73	0.0	226	0.8	28.3	11.5	19.5	61	17	35	17.8
Nov-2019	77.57	43.4	152	0.8	23.9	9.1	15.8	71	24	45	14.6
Dec-2019	46.03	76.7	105	0.9	17.4	6.7	11.5	81	40	63	12.0

ETo = Evapotranspiration; Precip = Precipitation; Sol Rad = solar radiation; Vap Pres = Vapor pressure; Temp = Temperature; Rel Hum = Relative humidity.

Table 3.2 (continued)

	Total		Average								
Month	ETo mm	Precip mm	Sol Rad W/m^2	Vap Pres kPa	Max Air Temp °C	Min Air Temp °C	Air Temp °C	Max Rel Hum %	Min Rel Hum %	Rel Hum %	Soil Temp °C
Jan-2020	67.31	2.3	142	0.8	19.5	6.5	12.5	79	34	56	11.3
Feb-2020	94.11	2.4	189	0.7	21.2	7.1	13.9	71	23	42	12.1
Mar-2020	93	99.6	193	1.0	18.6	8.2	13.0	88	43	65	15.1
Apr-2020	122.61	78.8	239	1.2	22.5	11.4	16.5	85	45	63	17.7
May-2020	184.08	0.0	323	1.3	27.9	13.7	20.2	84	35	57	22.3
Jun-2020	163.18	0.5	278	1.4	29.0	15.2	21.4	80	36	61	22.9
Jul-2020	207.54	0.0	340	1.4	33.1	16.4	24.2	79	26	49	25.0
Aug-2020	196.54	0.0	303	1.6	35.5	19.1	26.6	75	27	48	25.1
Sep-2020	160.75	0.1	249	1.3	35.1	17.1	25.4	71	21	43	22.8
Oct-2020	122.09	0.0	202	1.1	30.2	15.0	22.0	68	25	44	20.2
Nov-2020	81.71	0.2	159	0.7	24.0	8.6	15.8	69	23	42	14.7
Dec-2020	68.49	32.8	118	0.5	20.5	6.7	13.2	61	21	38	10.8
Jan-2021	75.79	41.4	147	0.6	19.7	6.3	12.8	70	28	47	10.5
Feb-2021	89.24	0.3	198	0.7	20.0	6.8	13.1	71	27	47	11.8
Mar-2021	118.28	28.9	244	0.8	19.8	6.3	12.7	80	32	54	13.1
Apr-2021	149.18	0.0	283	1.0	24.2	10.3	16.6	80	33	54	16.9
May-2021	163.79	0.0	306	1.3	25.1	12.5	17.8	87	39	62	20.0
Jun-2021	188.21	3.5	337	1.5	31.0	15.3	22.3	81	32	54	22.7
Jul-2021	205.85	3.0	328	1.6	33.7	18.5	25.3	78	29	50	24.5
Aug-2021	181.45	0.1	291	1.5	32.8	17.9	24.6	75	30	50	23.5
Sep-2021	148.96	0.3	260	1.4	31.3	16.1	22.9	81	31	53	22.4
Oct-2021	102.41	11.3	200	1.0	25.5	11.7	17.9	76	31	51	18.1
Nov-2021	84.03	0.1	166	0.9	25.4	10.3	17.0	71	25	47	16.1
Dec-2021	38.59	100	105	0.9	16.7	5.7	10.5	91	46	70	11.7
Jan-2022	76.55	3.0	142	0.6	20.1	7.1	13.3	65	23	40	10.8

ETo = Evapotranspiration; Precip = Precipitation; Sol Rad = solar radiation; Vap Pres = Vapor pressure; Temp = Temperature; Rel Hum = Relative humidity.

Data from each study were analyzed separately using linear mixed models with the lme4 package in R 4.4.0 (R Core Team 2020) where the date of rating each year was considered a fixed effect, while years and accession were considered random effects. Best linear unbiased predictions (BLUPs) were calculated for each trait and accession. Because BLUPs exhibit shrinkage towards the mean, thereby increasing prediction accuracy through a reduction in variance, BLUPs were calculated for all accessions in the original dataset for their respective studies to improve statistical power (Piepho et al. 2008; Piepho and Möhring 2006).

Genotyping and population structure

Tested accessions were sampled from field plots and grown in pots under greenhouse conditions for DNA extraction. DNA extraction was from leaf tissue using the Diversity Arrays Technologies recommended protocol (https://ordering.diversityarrays.com/files/DArT_DNA_isolation.pdf). DNA quality was assessed by gel electrophoresis on 1.5% agarose gels and concentrations were quantified using a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA). Samples diluted to a concentration of 100 ng/uL were loaded on 96-well plates and submitted to Diversity Arrays Technologies for DArTseq genotyping.

DArTseq generated both co-dominant (SNP) markers and dominant (silicoDArT) markers, though the latter were not analyzed in this study. DArTseq reduces genome complexity via restriction enzymes, producing short sequencing

reads. The SNP data from DArTseq was processed using the dartR package (Gruber et al. 2018) in R. Data were filtered by removing markers with less than 100% reproducibility, those that were monomorphic, and those with the call rate below 95% call rat, those with a minor allele frequency (MAF) less than 0.05, and those which shared a sequence tag (secondaries). Filtered SNPs were aligned to the reference contig assembly of the bermudagrass cultivar, 'Tifway' (Hulse-Kemp, [unpublished data] 2022) using BLAST (Madden 2013) with an E-value of 5×10^{-7} and a minimum sequence identity of 80%. 'Tifway' is a commercially available triploid hybrid between *Cynodon transvaalensis* x *Cynodon dactylon* developed in Tifton, GA, USA (Burton 1966). The contig assembly was divided into 650 contigs across the two genomes of 'Tifway'.

A distance matrix was calculated using the Euclidean distance method, then used to build a dendrogram based on the neighbor-joining (NJ) method (Saitou and Nei 1987) using the nj function available in R. The population structure was analyzed using STRUCTURE 2.3.4 (Pritchard, Stephens, and Donnelly 2000), with five runs for each value of *K* from 2 to 25 using a burn-in period of 10,000 and 20,000 Markov chain Monte Carlo (MCMC) iterations. Both the admixture model and correlated allele frequencies were assumed. Structure Harvester (Earl and vonHoldt 2012) was used to determine the most probable number of clusters (*K*) using the Evanno method. After determining the most likely *K*-value, an additional run was performed using the same parameters (i.e. admixture and correlated frequencies assumed), but higher burn-in and MCMC

iterations (100,000 and 200,000, respectively). Structure_threader (Pina-Martins et al. 2017) was used to parallelize and automate the analyses from STRUCTURE and Structure Harvester, and to visualize hierarchical clustering. Principal component analysis (PCA) was performed and visualized in dartR, and scatter plots were used to visualize clustering of accessions to confirm results from Structure Harvester.

The ploidy level for the collection accessions and commercial standard were known (see Chapter 1), but were unknown for the nursery accessions. To estimate their ploidy levels statistical analysis of the sequencing SNP data from DArTseq was performed using the gbs2ploidy package in R (Gompert and Mock 2017). This method infers cytotypes of individuals with unknown ploidy by estimating allelic proportions from heterozygous SNPs with different allelic ratios, then uses principal component analysis (PCA) and discriminant analysis (DA) to assign the probability of a specific cytotype to each accession. The optimal number of clusters for k-means clustering was determined using the NbClust package (Charrad et al. 2014) based on all available indices, and visualized using the factoextra package (Kassambara and Mundt 2020) in R. As ploidy levels of 26 collection accessions and 6 commercial standards were known, they were used to validate the results from gbs2ploidy. The linkage disequilibrium (LD) decay was calculated as pairwise comparisons using PLINK (Purcell et al. 2007).

Association mapping

A genome-wide association study (GWAS) was performed with the filtered SNP data from DArTseq using GEMMA (Zhou and Stephens 2012). The filtered SNP data was converted from a genlight object to the PLINK format for compatibility with GEMMA using the gl2plink function in dartR. The genotypes from DArTseq and phenotype BLUPs for VC were used to fit a univariate linear mixed model in GEMMA. The population structure (Q-matrix) determined by STRUCTURE at the best *K*-value, the standardized relatedness matrix determined by GEMMA, and the estimated ploidy level determined for each accession by gbs2ploidy were used as covariates in the model.

Results

Winter color retention

Summary statistics were calculated for each independent trial using their full datasets (Table S3.2) or a subset of the data that includes only accessions found in the present study (Table 3.3), herein referred to as the “full” and “selected” datasets, respectively. This was done to illustrate the amount of phenotypic variation in each population prior to their participation in this study. The distribution of the selected datasets was also plotted using ggplot2 in R (Wickham 2016) (Figure 3.1-2). When examining the means of the selected datasets, accessions from the 2017 trial had a higher overall mean VQ (5.256) than the other trials and the second highest overall mean VC (6.000), while

accessions from the 2019 NTEP test had a higher overall mean VC (6.056) and the second highest overall mean VQ (4.972). Across all studies, VQ and VC had a strong positive correlation at $p < 0.0001$ when using the Pearson's correlation coefficient (r). Correlations between VQ and VC were 0.75, 0.79, 0.71, and 0.81 for the germplasm collection, the 2018 nursery, the 2017 trial, and the 2019 NTEP trial, respectively. Correlations were also calculated for progenies of each maternal group, though correlations were weak. Correlations of the maternal groups ranged from r = -0.17 to 0.19. The maternal accession B131 had the highest BLUP for the winter color retention of all germplasm accessions tested (6.189), with similar performance from derived nursery accessions where the average BLUPs for progeny of B131 were VQ = 4.584 and VC = 5.947. Maternal accession B49 had the highest correlation with both VQ (r = 0.16) and VC (r = 0.19), though it was not present among the germplasm collection accessions, suggesting either mortality or contamination. The average BLUPs for progeny of B49 were 4.603 and 5.630 for VQ and VC, respectively.

Population structure and genetic marker analysis-

A total of 225,159 SNP markers were generated with the average reproducibility of 99.47% and the overall missing rate of 30.93% from the DArTseq platform. After filtering, 12,765 markers were retained for downstream analyses. The average call rate of these markers was 97.80% and the overall missing rate of 2.21%. The average minor allele frequency of the SNPs per locus

was 0.18. A total of 25 groups were analyzed in this study, with 23 corresponding to maternal lines for accessions from the 2018 nursery and two ‘unknown’ groups. The first unknown group, ‘U’, includes nursery accessions with unknown maternal or paternal contributions; possible errors with labeling or maternal accessions involved in open pollination. The second “unknown” group ‘N/A’ corresponds to germplasm accessions or commercial standards with no pedigree data. Though similar, accessions in the ‘U’ group have at least one parent from the UCR germplasm collection. Accessions in the ‘N/A’ group are further genetically isolated as they are derived from other countries of origin (germplasm) or other universities/companies (commercial standards).

Genetic distance matrices were calculated in dartR on the subpopulation and individual accession basis. The genetic distance between (maternal) subpopulations ranged from -0.097768 to 0.410903. A negative genetic distance is outside the natural range of the *F*-statistic values and may arise with unequal sample sizes observed between of two populations (Gerlach et al. 2010; Weir and Cockerham 1984). The lowest genetic distance was observed between a subpopulation derived from the germplasm collection maternal accession B91, and the ‘N/A’ group to which the other germplasm collection accessions and the commercial standards belong to. The highest genetic distance was observed between the subpopulations of B116 and B132. The range of genetic distances between individuals was between 0.026213 and 0.595085. The lowest genetic distance was between UCRC180576 and its internal replicate, while the highest

genetic distance was between UCRC180719 and the germplasm accession B121.

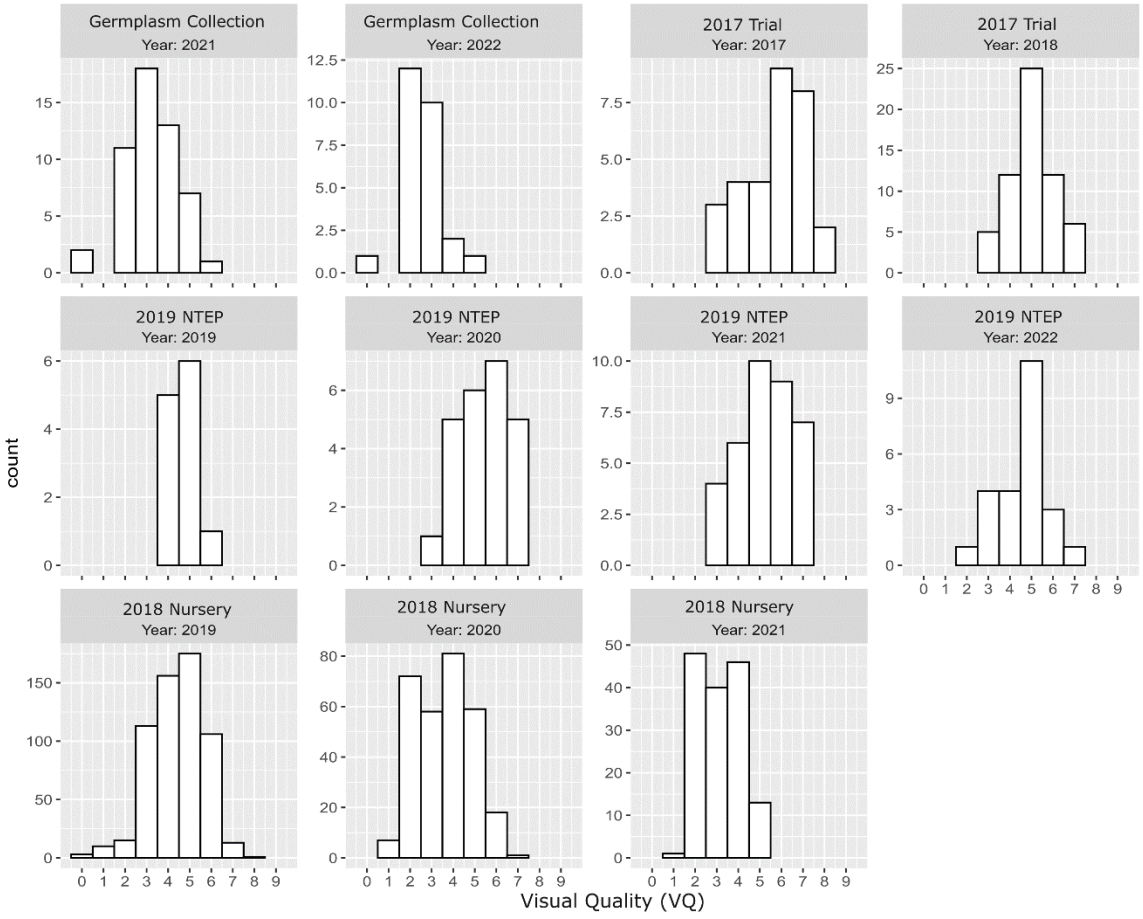

Figure 3.1 Distribution of mean visual quality (VQ) for each study and year in accessions used in this study. Visual quality was evaluated on a 1 to 9 scale, where 1 = poor or dead turf and 9 = ideal turf (Morris and Shearman 1998). If an accession could not be rated due to mortality, then it was assigned a 0 for visualization purposes.

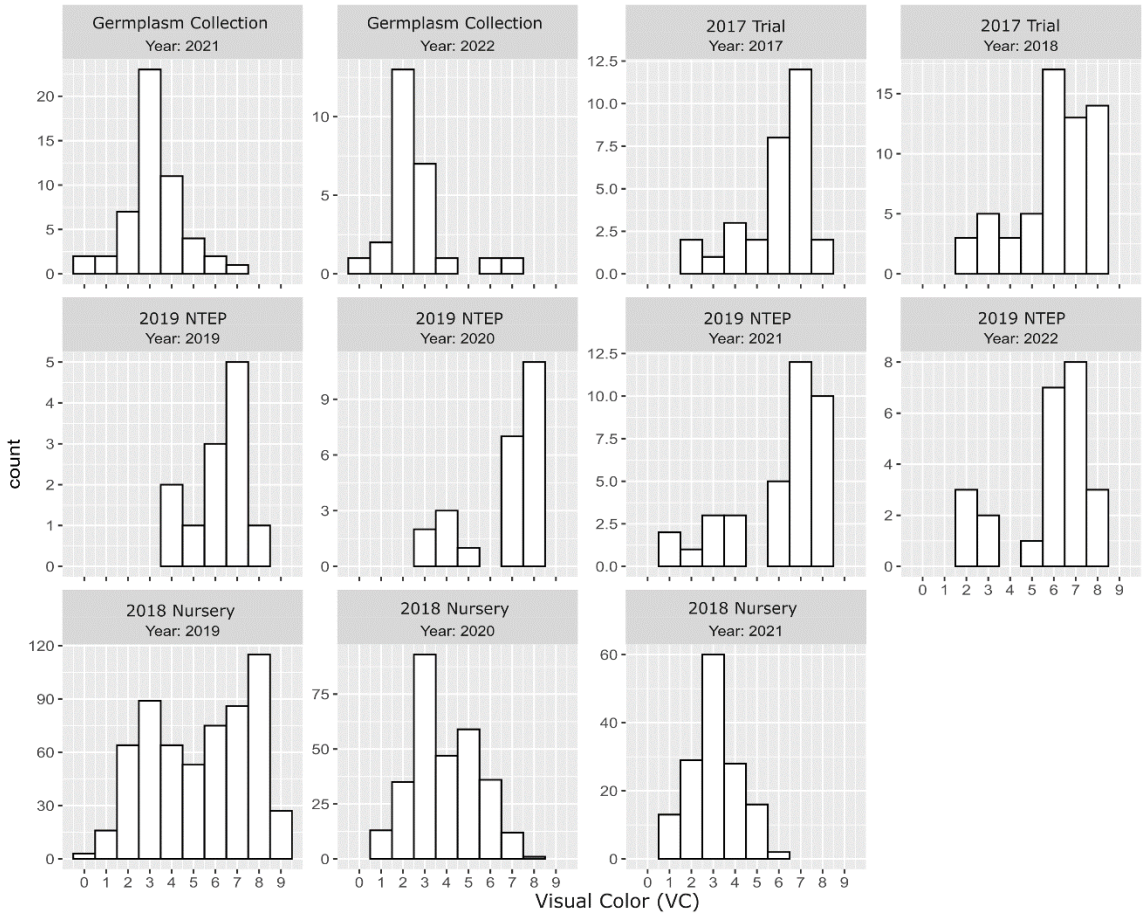

Figure 3.2 Distribution of mean visual color (retention) (VC) for each study and year in tested accessions. Visual color was evaluated on a 1 to 9 scale, where 1 = straw-brown color and 9 = dark green (Morris and Shearman 1998). Accession which could not be rated due to mortality, were assigned 0 for visualization purposes.

Table 3.3 Descriptive statistics of visual quality (VQ) and color (retention) (VC) across all rating dates for each study.

Date	Mean	min	max	SD	Mean	min	max	SD
Germplasm Collection		VQ				VC		
January 14, 2021	2.92	1.00	5.00	1.02	3.19	1.00	7.00	1.33
February 2, 2021	3.58	1.00	6.00	1.33	3.27	1.00	6.00	1.37
January 4, 2022	2.58	1.00	5.00	0.95	2.54	1.00	7.00	1.42
2018 Nursery		VQ				VC		
January 11, 2019	4.30	1.00	8.00	1.20	5.52	1.00	9.00	2.27
February 11, 2019	4.93	1.00	7.00	1.17	6.64	1.00	9.00	1.92
February 25, 2019	4.34	1.00	7.00	1.26	5.45	1.00	9.00	2.15
December 31, 2019	4.18	1.00	6.00	1.14	3.78	1.00	8.00	1.83
February 7, 2020	3.39	1.00	6.00	1.25	3.97	1.00	8.00	1.72
December 9, 2020	3.77	1.00	7.00	1.32	3.82	1.00	7.00	1.27
February 3, 2021	3.15	1.00	5.00	1.00	3.07	1.00	6.00	1.14
2017 Trial		VQ				VC		
December 18, 2017	6.20	4.00	8.00	1.32	6.20	4.00	7.00	1.08
December 29, 2017	5.20	3.00	7.00	1.42	5.60	2.00	8.00	1.99
January 19, 2018	4.87	3.00	7.00	1.06	5.60	2.00	8.00	2.10
February 1, 2018	5.20	4.00	6.00	0.77	6.33	3.00	8.00	1.76
February 16, 2018	5.73	4.00	7.00	1.10	7.13	6.00	8.00	0.83
February 26, 2018	4.33	3.00	6.00	0.90	5.13	3.00	7.00	1.46
2019 NTEP		VQ				VC		
December 30, 2019	4.67	4.00	6.00	0.65	6.17	4.00	8.00	1.27
February 6, 2020	5.33	4.00	7.00	1.07	6.75	4.00	8.00	1.54
December 10, 2020	5.50	3.00	7.00	1.31	6.58	3.00	8.00	2.02
January 31, 2021	5.00	3.00	7.00	1.13	5.33	1.00	8.00	2.50
December 13, 2021	5.00	3.00	7.00	1.21	6.08	3.00	8.00	1.73
January 4, 2022	4.33	2.00	7.00	1.30	5.42	2.00	8.00	2.15

Based on the Evanno method as implemented by Structure Harvester, the most probable number of clusters (*K*) was determined to be $K = 7$. Individuals sharing a maternal accession were likely to group together, assuming high heritability of a character (Figure 3.3). One accession, UCRC180175, did not group with its replicate, signaling a possible contamination or error during sampling. The remaining replicated accessions grouped as expected. Clear

groupings could not be distinguished among the population based on the PCA (Figure 3.4), with only 17.1% and 8.3% of the variability explained by principal components 1 and 2, respectively.

Bermudagrass is a self-incompatible perennial grass expected to have high heterozygosity. Observed and expected heterozygosity, as well as inbreeding coefficients, were calculated with dartR per maternal subpopulation. Observed heterozygosity ranged from 0.16 to 0.34, while the expected heterozygosity ranged from 0.08 to 0.30. Inbreeding coefficients ranged from -0.49 to 0.25, suggesting an excessive amount of heterozygotes. Estimates of LD were quite low and had a mean of $r^2 = 0.05$ and a median of $r^2 = 0.04$.

In silico ploidy estimation-

The gbs2ploidy package was able to assign a ploidy level to most accessions at a high estimated probability. Principal component analysis (PCA) and discriminant analysis (DA) were used to assign each accession to its estimated cytotype. Cytotype probabilities were calculated using a range of principal components from two to six. The most optimal number of principal components was determined by k-means clustering to be five, thus the cytotype probabilities for each accession were based on principal components one through five. Prior to *in silico* ploidy estimations, the ploidy levels for 32 of the 179 accessions were known. Ploidy estimates by gbs2ploidy mostly

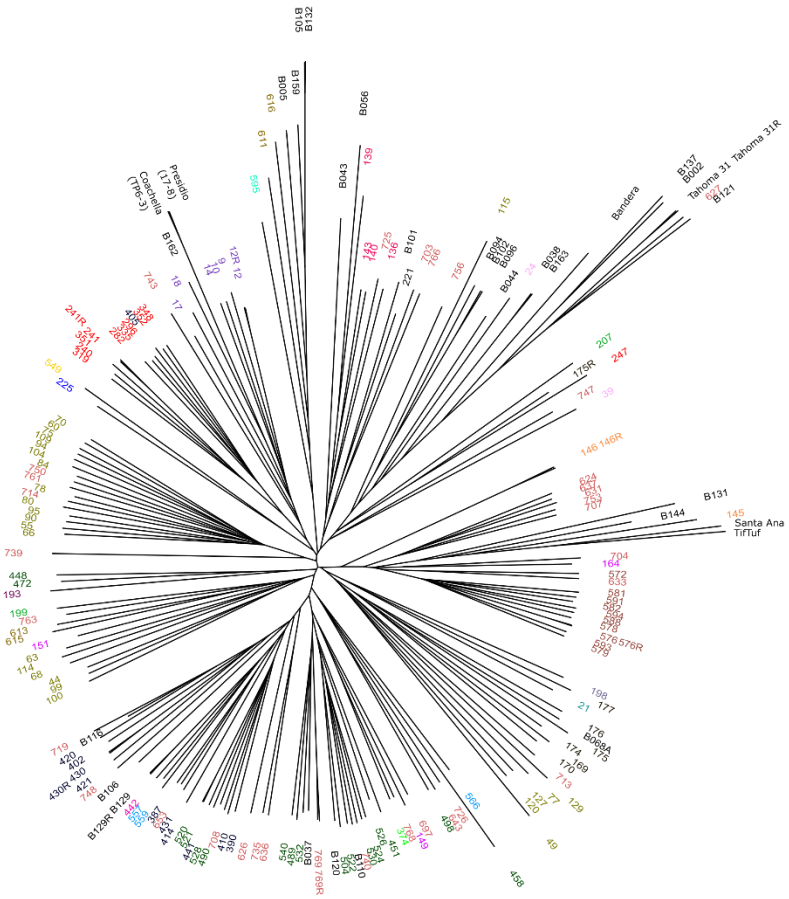

Figure 3.3 Dendrogram based on the Neighbor-joining (NJ) method and SNP markers for 179 accessions from four separate studies. Accessions in the same color share a common maternal parent. Accessions in black have unknown pedigree data. To improve readability, nursery accessions with the prefix “UCRC18” were reduced to their numerical identifier without leading zeros (e.g., UCRC180009 = 9).

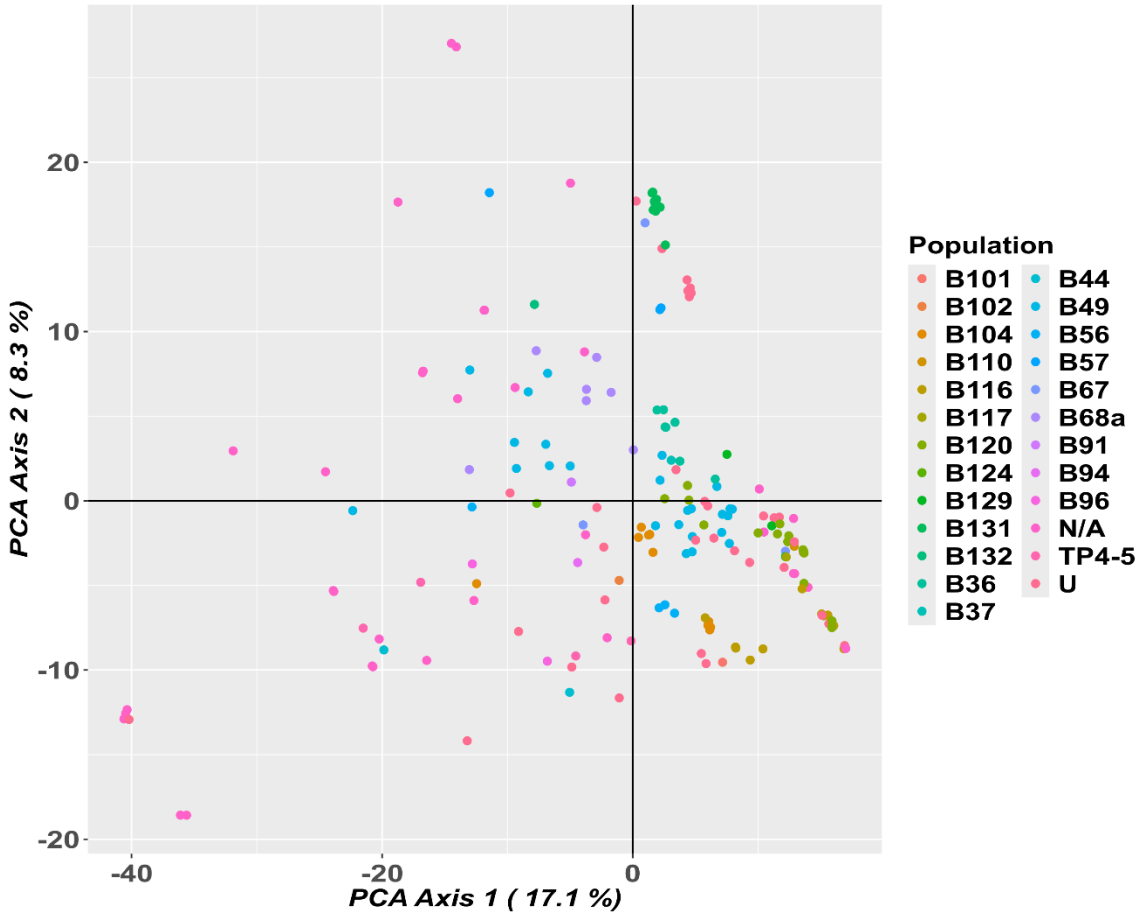

Figure 3.4 Principal component analysis (PCA) describing the population structure of accessions present in the study. Accessions sharing the same color belong to the same maternal subpopulation.

matched these known values, though eight germplasm accessions (B037, B110, B116, B120, B129, B131, B159, and B162) and one commercial standard ('Bandera') were assigned to a different cytotype than determined by chromosome counts (Table 3.4). Some of these accessions are maternal lines for several accessions in the 2018 bermudagrass nursery, and differences between the previously known chromosome numbers and the ploidy levels estimated here make it difficult to interpret the results. For example, chromosome

counts of B129 identified it as a tetraploid and as such, its progeny could only be tetraploid or triploid. However, B129 was identified *in silico* with a high probability as diploid. Estimated ploidy levels of the B129 progeny appear to support the diploid level, as they are estimated to be either diploid or triploid. Clearly, chromosome counts of this accession must be revisited. *In silico* ploidy estimation identified 40 diploids, 74 triploids, and 33 tetraploids among the bermudagrass hybrids in the 2018 nursery. No higher ploidy levels were assumed or expected, knowing the chromosome numbers of accessions within the germplasm collection. While there is some potential for unreduced gametes, no such possibility was assumed in this study.

Several accessions were assigned to two cytotypes at similar probabilities, typically ranging between 41–59% for each group. These the expected types based on prior maternal ploidy information. In such cases, the higher probability was chosen. A total of eight such accessions were observed with uncertain assignments. Among the 147 nursery accessions, 33 were of unknown maternal contribution. No assumptions could be made for these accessions regarding the reliability of the *in silico* ploidy estimation. Of the nine replicates used for internal validation, two were estimated to have a different assignment from their

Table 3.4 Estimated ploidy levels as determined by gbs2ploidy. The probability of an accession belonging to a specific cytotype is listed below, along with their assigned maternal subpopulation and the expected ploidy levels that could be derived from the maternal line.

	Cytotype probability			Maternal		Expected		
Accession	4x	2x	3x	Subpopulation	Ploidy	Ploidy A	Ploidy B	Estimated Ploidy
Bandera	1.00	0.00	0.00	N/A[b]		3		4
Coachella	0.10	0.00	0.90	N/A		3		3
Presidio	0.00	0.08	0.92	N/A		3		3
Santa Ana	0.00	0.23	0.77	N/A		3		3
Tahoma 31	0.01	0.01	0.99	N/A		3		3
Tahoma 31R[a]	0.00	0.11	0.89	N/A		3		3
TifTuf	0.00	0.21	0.79	N/A		3		3
B002	0.00	0.84	0.16	N/A	2	2		2
B005	0.00	0.01	0.99	N/A	3	3		3
B037	0.00	0.64	0.36	N/A	4	4		2
B038	1.00	0.00	0.00	N/A	4	4		4
B043	0.00	1.00	0.00	N/A	2	2		2
B044	1.00	0.00	0.00	N/A	4	4		4
B056	0.00	1.00	0.00	N/A	4	4		2
B068A	0.99	0.00	0.01	N/A	4	4		4
B094	1.00	0.00	0.00	N/A	4	4		4
B096	1.00	0.00	0.00	N/A	4	4		4
B101	0.01	0.01	0.99	N/A	4	4		3
B102	1.00	0.00	0.00	N/A	4	4		4
B105	0.00	1.00	0.00	N/A	2	2		2
B106	0.00	0.14	0.86	N/A	4	4		3
B110	0.00	1.00	0.00	N/A	4	4		2
B116	0.00	0.99	0.01	N/A	4	4		2
B120	0.00	0.99	0.01	N/A	4	4		2
B121	0.03	0.00	0.96	N/A	3	3		3
B129	NaN[c]	NaN	NaN	N/A	4	4		2
B129R	0.00	1.00	0.00	N/A	4	4		2
B131	0.00	0.55	0.45	N/A	4	4		2
B132	0.00	1.00	0.00	N/A	2	2		2
B137	0.00	0.89	0.11	N/A	2	2		2

[a] R = Replicate; [b] N/A = Not applicable; [c] NaN = Not a number (probability not calculated).

Table 3.4 (continued).

	Cytotype probability			Maternal		Expected		
Accession	4x	2x	3x	Subpopulation	Ploidy	Ploidy A	Ploidy B	Estimated Ploidy
B144	0.00	0.04	0.96	N/A[b]	3	3		3
B159	0.00	1.00	0.00	N/A	3	3		2
B162	0.00	0.02	0.98	N/A	4	4		3
B163	1.00	0.00	0.00	N/A	4	4		4
UCRC180009	0.00	0.80	0.20	B36	4	4	3	2
UCRC180010	0.00	0.03	0.97	B36	4	4	3	3
UCRC180012	0.00	0.01	0.99	B36	4	4	3	3
UCRC180012R[a]	0.00	0.12	0.88	B36	4	4	3	3
UCRC180014	0.00	0.03	0.97	B36	4	4	3	3
UCRC180017	0.00	0.02	0.98	B36	4	4	3	3
UCRC180018	0.00	0.33	0.67	B36	4	4	3	3
UCRC180021	0.00	0.01	0.99	B36	4	4	3	3
UCRC180024	1.00	0.00	0.00	B44	4	4	3	4
UCRC180039	0.09	0.00	0.91	B44	4	4	3	3
UCRC180044	0.00	0.01	0.99	B49	4	4	3	3
UCRC180049	0.99	0.00	0.01	B49	4	4	3	4
UCRC180055	0.09	0.00	0.91	B49	4	4	3	3
UCRC180060	0.79	0.00	0.21	B49	4	4	3	4
UCRC180063	1.00	0.00	0.00	B49	4	4	3	4
UCRC180066	0.02	0.00	0.98	B49	4	4	3	3
UCRC180068	1.00	0.00	0.00	B49	4	4	3	4
UCRC180070	0.93	0.00	0.07	B49	4	4	3	4
UCRC180075	0.78	0.00	0.22	B49	4	4	3	4
UCRC180077	1.00	0.00	0.00	B49	4	4	3	4
UCRC180078	0.00	0.01	0.98	B49	4	4	3	3
UCRC180080	0.01	0.00	0.98	B49	4	4	3	3
UCRC180084	0.01	0.01	0.99	B49	4	4	3	3
UCRC180090	0.00	0.01	0.99	B49	4	4	3	3
UCRC180094	1.00	0.00	0.00	B49	4	4	3	4
UCRC180095	0.01	0.01	0.99	B49	4	4	3	3
UCRC180099	0.07	0.00	0.93	B49	4	4	3	3
UCRC180100	0.00	0.01	0.98	B49	4	4	3	3
UCRC180104	0.06	0.00	0.94	B49	4	4	3	3

[a] R = Replicate; [b] N/A = Not applicable; [c] NaN = Not a number (probability not calculated).

Table 3.4 (continued)

	Cytotype probability			Maternal		Expected		
Accession	4x	2x	3x	Subpopulation	Ploidy	Ploidy A	Ploidy B	Estimated Ploidy
UCRC180109	0.55	0.00	0.45	B49	4	4	3	4
UCRC180114	0.96	0.00	0.04	B49	4	4	3	4
UCRC180115	1.00	0.00	0.00	B49	4	4	3	4
UCRC180120	0.99	0.00	0.01	B49	4	4	3	4
UCRC180127	0.99	0.00	0.01	B49	4	4	3	4
UCRC180129	0.07	0.00	0.93	B49	4	4	3	3
UCRC180136	0.00	0.21	0.79	B56	4	4	3	3
UCRC180139	0.00	0.74	0.26	B56	4	4	3	2
UCRC180140	0.00	0.58	0.42	B56	4	4	3	3
UCRC180143	0.00	0.28	0.72	B56	4	4	3	3
UCRC180145	0.00	0.04	0.95	B57	4			3
UCRC180146	1.00	0.00	0.00	B57	4			4
UCRC180146R[a]	0.00	0.24	0.76	B57	4			3
UCRC180149	0.00	1.00	0.00	B67	2	2	3	2
UCRC180151	0.00	1.00	0.00	B67	2	2	3	2
UCRC180164	0.17	0.00	0.83	B67	2	2	3	3
UCRC180169	0.03	0.00	0.97	B68a	4	4	3	3
UCRC180170	0.59	0.00	0.41	B68a	4	4	3	4
UCRC180174	0.03	0.00	0.97	B68a	4	4	3	3
UCRC180175	0.03	0.00	0.96	B68a	4	4	3	3
UCRC180175R	0.00	0.40	0.60	B68a	4	4	3	3
UCRC180176	0.07	0.00	0.93	B68a	4	4	3	3
UCRC180177	0.00	0.02	0.98	B68a	4	4	3	3
UCRC180193	0.99	0.00	0.01	B91	4	4	3	4
UCRC180198	0.20	0.00	0.80	B94	4	4	3	3
UCRC180199	1.00	0.00	0.00	B96	4	4	3	4
UCRC180207	0.01	0.01	0.98	B96	4	4	3	3
UCRC180221	0.00	0.20	0.80	B101	4	4	3	3
UCRC180225	0.01	0.00	0.99	B102	4	4	3	3
UCRC180240	0.00	0.06	0.94	B104	4	4	3	3
UCRC180241	0.00	0.09	0.91	B104	4	4	3	3
UCRC180241R	0.00	0.62	0.38	B104	4	4	3	2
UCRC180247	0.00	0.09	0.91	B104	4	4	3	3

[a] R = Replicate; [b] N/A = Not applicable; [c] NaN = Not a number (probability not calculated).

Table 3.4 (continued)

Accession	Cytotype probability 4x	2x	3x	Maternal Subpopulation	Ploidy	Expected Ploidy A	Ploidy B	Estimated Ploidy
UCRC180282	0.00	0.04	0.96	B104	4	4	3	3
UCRC180296	0.00	0.31	0.69	B104	4	4	3	3
UCRC180319	0.00	0.07	0.93	B104	4	4	3	3
UCRC180335	0.00	0.38	0.62	B104	4	4	3	3
UCRC180348	0.00	0.11	0.89	B104	4	4	3	3
UCRC180351	0.00	0.37	0.63	B104	4	4	3	3
UCRC180352	0.00	0.09	0.91	B104	4	4	3	3
UCRC180374	0.00	0.98	0.02	B110	4	4	3	2
UCRC180387	0.00	0.98	0.02	B116	4	4	3	2
UCRC180390	0.00	1.00	0.00	B116	4	4	3	2
UCRC180402	0.00	0.99	0.01	B116	4	4	3	2
UCRC180405	0.00	0.95	0.05	B116	4	4	3	2
UCRC180410	0.00	1.00	0.00	B116	4	4	3	2
UCRC180414	0.00	1.00	0.00	B116	4	4	3	2
UCRC180420	0.00	1.00	0.00	B116	4	4	3	2
UCRC180421	0.00	0.94	0.06	B116	4	4	3	2
UCRC180430	0.00	0.56	0.44	B116	4	4	3	2
UCRC180430R[a]	0.00	0.89	0.11	B116	4	4	3	2
UCRC180431	0.00	1.00	0.00	B116	4	4	3	2
UCRC180441	0.00	0.99	0.01	B116	4	4	3	2
UCRC180442	0.00	1.00	0.00	B117	4	4	3	2
UCRC180448	0.00	0.03	0.97	B120	4	4	3	3
UCRC180451	0.00	0.94	0.06	B120	4	4	3	2
UCRC180458	0.00	1.00	0.00	B120	4	4	3	2
UCRC180472	0.00	0.02	0.98	B120	4	4	3	3
UCRC180489	0.00	0.94	0.06	B120	4	4	3	2
UCRC180490	0.00	0.99	0.01	B120	4	4	3	2
UCRC180498	0.00	0.17	0.83	B120	4	4	3	3
UCRC180504	0.00	1.00	0.00	B120	4	4	3	2
UCRC180520	0.00	0.98	0.02	B120	4	4	3	2
UCRC180521	0.00	0.95	0.05	B120	4	4	3	2
UCRC180522	0.00	1.00	0.00	B120	4	4	3	2
UCRC180524	0.00	0.99	0.01	B120	4	4	3	2

[a] R = Replicate; [b] N/A = Not applicable; [c] NaN = Not a number (probability not calculated).

Table 3.4 (continued)

	Cytotype probability			Maternal		Expected		
Accession	4x	2x	3x	Subpopulation	Ploidy	Ploidy A	Ploidy B	Estimated Ploidy
UCRC180526	0.00	0.99	0.01	B120	4	4	3	2
UCRC180528	0.00	0.99	0.01	B120	4	4	3	2
UCRC180530	0.00	0.99	0.01	B120	4	4	3	2
UCRC180532	0.00	1.00	0.00	B120	4	4	3	2
UCRC180540	0.00	0.92	0.08	B120	4	4	3	2
UCRC180549	0.85	0.00	0.15	B124	3	3		4
UCRC180557	0.00	1.00	0.00	B129	4	4	3	2
UCRC180559	0.00	0.97	0.03	B129	4	4	3	2
UCRC180566	0.00	0.10	0.90	B129	4	4	3	3
UCRC180572	0.98	0.00	0.02	B131	4	4	3	4
UCRC180576	0.67	0.00	0.33	B131	4	4	3	4
UCRC180576R[a]	0.98	0.00	0.02	B131	4	4	3	4
UCRC180578	0.03	0.00	0.97	B131	4	4	3	3
UCRC180579	0.04	0.00	0.96	B131	4	4	3	3
UCRC180581	0.72	0.00	0.28	B131	4	4	3	4
UCRC180582	0.02	0.00	0.98	B131	4	4	3	3
UCRC180588	0.05	0.00	0.95	B131	4	4	3	3
UCRC180591	0.94	0.00	0.06	B131	4	4	3	4
UCRC180593	0.00	0.03	0.97	B131	4	4	3	3
UCRC180594	0.83	0.00	0.17	B131	4	4	3	4
UCRC180595	0.00	0.21	0.79	B132	4	4	3	3
UCRC180611	0.02	0.00	0.98	TP4-5	N/A[b]			3
UCRC180613	0.00	0.08	0.91	TP4-5	N/A			3
UCRC180615	0.06	0.00	0.93	TP4-5	N/A			3
UCRC180616	0.00	0.01	0.98	TP4-5	N/A			3
UCRC180624	0.81	0.00	0.19	U				4
UCRC180626	0.00	0.47	0.53	U				3
UCRC180627	0.01	0.00	0.98	U				3
UCRC180631	0.54	0.00	0.46	U				4
UCRC180633	0.03	0.00	0.97	U				3
UCRC180636	0.00	0.08	0.92	U				3
UCRC180637	0.29	0.00	0.71	U				3
UCRC180643	0.01	0.01	0.99	U				3

[a] R = Replicate; [b] N/A = Not applicable; [c] NaN = Not a number (probability not calculated).

Table 3.4 (continued)

Accession	Cytotype probability 4x	2x	3x	Maternal Subpopulation	Ploidy	Expected Ploidy A	Ploidy B	Estimated Ploidy
UCRC180653	0.00	1.00	0.00	U				2
UCRC180697	0.00	0.99	0.01	U				2
UCRC180703	0.02	0.00	0.98	U				3
UCRC180704	0.05	0.00	0.95	U				3
UCRC180707	0.65	0.00	0.35	U				4
UCRC180708	0.00	0.99	0.01	U				2
UCRC180713	0.78	0.00	0.22	U				4
UCRC180714	0.02	0.00	0.98	U				3
UCRC180719	0.00	1.00	0.00	U				2
UCRC180725	0.09	0.00	0.91	U				3
UCRC180726	0.01	0.01	0.99	U				3
UCRC180735	0.00	0.23	0.77	U				3
UCRC180739	0.95	0.00	0.05	U				4
UCRC180740	0.00	1.00	0.00	U				2
UCRC180743	0.00	0.25	0.75	U				3
UCRC180747	0.64	0.00	0.36	U				4
UCRC180748	0.00	0.16	0.84	U				3
UCRC180750	0.01	0.01	0.99	U				3
UCRC180753	0.86	0.00	0.14	U				4
UCRC180756	1.00	0.00	0.00	U				4
UCRC180761	0.51	0.00	0.49	U				4
UCRC180763	0.00	0.03	0.97	U				3
UCRC180766	0.00	0.28	0.72	U				3
UCRC180768	0.00	0.99	0.01	U				2
UCRC180769	0.00	0.86	0.14	U				2
UCRC180769R[a]	0.00	0.64	0.36	U				2

[a] R = Replicate; [b] N/A = Not applicable; [c] NaN = Not a number (probability not calculated).

counterparts (UCRC180146 and UCRC180241). One entry of UCRC180146 was estimated to be tetraploid, while its replicate was estimated to be triploid. The first estimate, however, appears more certain (100% versus 76%, respectively). Similarly, UCRC180241 was estimated to be triploid (91%), while its replicate was estimated to be diploid (62%). In both cases, the cytotype with the higher probability was selected as the “correct” ploidy for this study.

GWAS findings

Genome-wide associations for winter color retention were attempted with 12,765 SNP markers using a univariate linear mixed model in GEMMA with the population structure, kinship, and ploidy estimates used as covariates. Two markers, snp.51109120 and snp.8463932, were significant when applying a threshold of $p < 1e\text{-}5$ ($p = 2.11e\text{-}6$ and $2.30e\text{-}6$, respectively) (Figure 3.5). Snp.8463932 is in contigs Sci67kf_39;HRSCAF=72 and ScybdfH_43;HRSCAF=84, where each contig belongs to one of the ‘Tifway’ sub-genomes. Using bedtools, 300 bp upstream and downstream of the marker sequence were taken from each sub-genome as sequence context for alignment via BLAST against the *Zea mays* reference genome (Zm-B73-REFERENCE-NAM-5.0 reference Annotation Release 103). Snp.51109120 did not align to the ‘Tifway’ contig assembly, so the sequence context could not be provided for alignments. Alignment of Snp.8463932 (29 bp + 600 bp) with the sequence context from Sci67kf_39;HRSCAF=72 (1x sub-genome of ‘Tifway’) yielded a

single result that included the full marker sequence. Snp.8463932 aligned to LOC100383765 on chromosome 1 of maize, which encodes a glucose-6-phosphate 1-dehydrogenase (G6PDH). Alignment using the sequence context from ScybdfH_43;HRSCAF=84 (2x sub-genome of 'Tifway') did not yield any notable results. Snp.51109120 aligned to the maize reference genome assembly without providing any sequence context. There were many successful alignments, but only the alignment with the lowest E-score (0.014) was considered. Snp.51109120 aligned to LOC100193781 on chromosome 6, which has been annotated as encoding a heparanase-like protein 3. Heparanase-like proteins are cell wall proteins, though the heparanase-like protein 3 has not so far been found implicated in stress response in maize (Niu and Wang 2020).

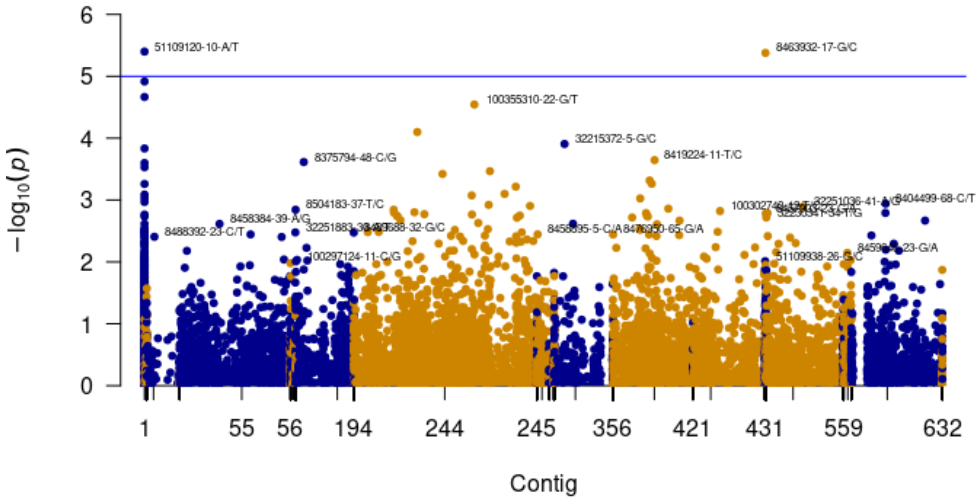

Figure 3.5 Visualization of SNP markers at an assigned threshold of *p* < 1e-5. Contigs were sorted alphabetically then reassigned a number 1 to 651 for visualization only. This does not imply that the contigs (visualized here or otherwise discussed) are physically sorted based on this number.

Discussion

Enhanced winter color retention is a highly desirable trait for perennial grasses used as either forage or turf. The ultimate goal for bermudagrass is to keep the grass green all year long. This could be achieved by either a very short dormancy period (requiring early spring green-up and/or delayed dormancy onset hence increased color retention) or no dormancy period at all. No dormancy means color retention and cold resistance through winter months. At the physiological level, the breakdown of chlorophyll and other proteins is tied with leaf senescence and the onset of dormancy, especially in response to abiotic stresses such as cold stress (Fagerness and Yelverton 2000). This phenotype of enhanced color retention, often referred to as stay-green (or staygreen) in other plant species, is characterized by delayed or reduced senescence of leaf tissues and is positively associated with biomass production (Thomas and Ougham 2014; Xu et al. 2019). In this study, visual color data recorded during the winter months was synonymous with winter color retention. Spring green-up data was not available in this study, but has been shown previously to be positively correlated with color retention in bermudagrass (Guo et al. 2017). Because cold tolerance is also associated with some morphological traits, turf quality was not included in the model used for genome-wide associations in this study. Although not explicitly addressed, quality data was not included in other genetic studies pertaining to stress tolerance (Bushman et al. 2024; Yu et al. 2022). Turf quality

is a subjective visual assessment of several functional traits at once, facilitating rapid screening of accessions (Morris and Shearman 1998). While it may have a positive correlation with color retention as found in this study, the objective of this study was to identify potential genetic markers associated with winter color retention.

Variations in climate conditions were apparent between years (Table 3.2) and affected population means (Figure 3.1-2). Mean VC appeared to be higher among the nursery accessions during the first two winters and decreasing significantly thereafter. This may be a consequence of higher plant vigor in the first year after establishment. In contrast, the distribution of accession responses in the 2019 NTEP test did not appear to change dramatically between years. This may be due to either warmer temperatures, slowing the dormancy onset and enabling acclimation to the minor cold stress, or due to experimental conditions imposed on the plants during the summer season. As previously described, accessions in the 2019 NTEP test were subjected to drought stress during summer months. Drought stress prior to the winter months increases the risk that plants fail to fully recover from drought-related injuries, and could be a factor in reduced quality and cold tolerance (Yu et al. 2023).

Significant associations of the SNP markers with visual ratings of plot appearance were sparse. Perhaps this implies a relatively simple genetic control of the character (such as very few loci with major effects) or it indicates a lack of statistical power in the GWAS as used here. There is no question that the

population structure here did not meet standard practices for GWAS studies. Normally, admixed populations (Figure 3.4) or populations with various ploidy levels (Table 3.4) are not desirable for genetic studies as both may introduce spurious associations as a consequence of differences in allele frequencies (Alseekh et al. 2021; Hellwege et al. 2017; Panarella and Burkett 2019). Here, a mixed population consisting of diploids, triploids, and tetraploids was analyzed, and the population structure showed high levels of heterozygosity. Different ploidy levels introduce differences in allele dosages and multiple alleles at a given loci are likely (Dufresne et al. 2014). In an attempt to mitigate the problem as much as possible, ploidy levels were included in the GWAS as a covariate. Estimation of ploidy levels *in silico* appeared successful in this study, even though chromosome counts or flow cytometry would be preferred as more direct methods, and have been widely used in bermudagrass studies (Karaca et al. 2000; Taliaferro et al. 1997). Although discrepancies exist between our prior ploidy knowledge and the new estimations, this method appears applicable to populations derived from open-pollination where the paternal contribution is unknown. There were a few discrepancies and it is uncertain what might have been the cause. While mistakes in sample collections or precise counts of very small chromosomes cannot be excluded, one could argue that DNA quality at the time of sequencing may play a role. Lower quality DNA or fragmented DNA could artificially lower the read counts at each SNP marker and potentially bias reads from one sub-genome over another, thereby reducing (or increasing) allelic

ratios. Regardless, chromosome counts of questionable accessions must be revisited.

A completed annotated genome assembly of bermudagrass was not available for this study. Recent literature reports the creation of such a reference genome for *Cynodon transvaalensis*, but it does not seem to be publicly available at this time (Cui et al. 2021). Given the absence of an accessible standard genome assembly for bermudagrass it may not be entirely surprising that only two SNP markers, snp.51109120 and snp.8463932, associated with the phenotypic characteristics, but at highly significant levels. Snp.51109120 did not map to the 'TifWay' contig assembly, and so no sequence context could be drawn for alignments against other related species. With BLAST, snp.8463932 aligned to the maize reference genome and mapped to LOC100383765. Maize was chosen for alignments for its well-annotated reference genome for a grass species. LOC100383765 in maize encodes *ZmG6PDH,* an enzyme known to be associated with stress responses. G6PDH is a key enzyme in the regulation of the pentose phosphate pathway that produces nicotinamide adenine dinucleotide phosphate (NADPH), essential for maintaining redox homeostasis and lipid biosynthesis (Esposito 2016). Recently, *ZmG6PDHs* have been found to enhance cold tolerance of maize through mitigation of the oxidative damage caused by ROS (Li et al. 2023). This outcome of the analysis here seems promising, but it certainly requires additional analyses to validate and use in practical breeding.

Conclusion

Winter color retention is an important consumer-related trait for bermudagrass and is a major target for breeding programs hoping to expand its geographical distribution. In this study, significant admixture was observed in our population, and attempts were made to address several conflicts due to the population structure and using various statistical methods. However, the use of such methods cannot guarantee the complete removal of bias or spurious results brought about by the population structure. Given the population structure in this study, GWAS was expected to be indicative but not highly informative. However, the results identified highly significant associations between two SNP markers and phenotypic variables associated with winter color retention. Both aligned to the maize reference genome; one indicated the role of a gene known to be involved in stress tolerance. Additional steps need to be taken to validate these findings and to confirm the suitability of such markers for marker-assisted selection.

References

Ahring, R. M., W. W. Huffine, C. M. Taliaferro, and R. D. Morrison. 1975. “Stand Establishment of Bermudagrass from Seed1.” *Agronomy Journal* 67(2):229–32. doi: 10.2134/agronj1975.00021962006700020014x.

Alseekh, Saleh, Dimitrina Kostova, Mustafa Bulut, and Alisdair R. Fernie. 2021. “Genome-Wide Association Studies: Assessing Trait Characteristics in Model and Crop Plants.” *Cellular and Molecular Life Sciences: CMLS* 78(15):5743–54. doi: 10.1007/s00018-021-03868-w.

Bertrand, Annick, Yves Castonguay, Aïda Azaiez, and Julie Dionne. 2013. “Low-Temperature Stress.” Pp. 279–318 in *Turfgrass: Biology, Use, and Management*. John Wiley & Sons, Ltd.

Breuninger, J. M., Welterlen, M. S., Augustin, B. J., Cline, V., & Morris, K. (2013). The Turfgrass Industry. In *Turfgrass: Biology, Use, and Management* (pp. 37–103). https://doi.org/10.2134/agronmonogr56.c2

Burton, Glenn W. 1966. “Tifway (Tifton 419) Bermudagrass (Reg. No. 7).”

Bushman, Bradley, Matthew Robbins, Yinjie Qiu, Eric Watkins, Andrew Hollman, Nicole Mihelich, Dominic Petrella, Florence Breullin-Sessoms, Ming-Yi Chou, and Paul Koch. 2024. “Association of Hard Fescue (Festuca Brevipila) Stress Tolerances with Genome Mapped Markers.” *Crop Science* 64(2):1002–14. doi: 10.1002/csc2.21155.

Casler, M. D., & Duncan, R. R. (2003). *Turfgrass Biology, Genetics, and Breeding*. John Wiley & Sons.

Charrad, Malika, Nadia Ghazzali, Véronique Boiteau, and Azam Niknafs. 2014. “NbClust: An R Package for Determining the Relevant Number of Clusters in a Data Set.” *Journal of Statistical Software* 61(6):1–36. doi: 10.18637/jss.v061.i06.

Cheng, Zhangmin, Rui Jin, Minjie Cao, Xiaodong Liu, and Zhulong Chan. 2016. “Exogenous Application of ABA Mimic 1 (AM1) Improves Cold Stress Tolerance in Bermudagrass (Cynodon Dactylon).” *Plant Cell, Tissue and Organ Culture (PCTOC)* 125(2):231–40. doi: 10.1007/s11240-016-0941-5.

Cui, Fengchao, Geli Taier, Manli Li, Xiaoxia Dai, Nan Hang, Xunzhong Zhang, Xiangfeng Wang, and Kehua Wang. 2021. “The Genome of the Warm-Season Turfgrass African Bermudagrass (Cynodon Transvaalensis).” *Horticulture Research* 8(1):1–16. doi: 10.1038/s41438-021-00519-w.

Dufresne, France, Marc Stift, Roland Vergilino, and Barbara K. Mable. 2014. “Recent Progress and Challenges in Population Genetics of Polyploid Organisms: An Overview of Current State-of-the-Art Molecular and Statistical Tools.” *Molecular Ecology* 23(1):40–69. doi: 10.1111/mec.12581.

Earl, Dent A., and Bridgett M. vonHoldt. 2012. “STRUCTURE HARVESTER: A Website and Program for Visualizing STRUCTURE Output and Implementing the Evanno Method.” *Conservation Genetics Resources* 4(2):359–61. doi: 10.1007/s12686-011-9548-7.

Esmaili, Somayeh, and Hassan Salehi. 2012. “Effects of Temperature and Photoperiod on Postponing Bermudagrass (Cynodon Dactylon [L.] Pers.) Turf Dormancy.” *Journal of Plant Physiology* 169(9):851–58. doi: 10.1016/j.jplph.2012.01.022.

Esposito, Sergio. 2016. “Nitrogen Assimilation, Abiotic Stress and Glucose 6-Phosphate Dehydrogenase: The Full Circle of Reductants.” *Plants* 5(2):24. doi: 10.3390/plants5020024.

Fagerness, M. J., and F. H. Yelverton. 2000. “Tissue Production and Quality of ‘Tifway’ Bermudagrass as Affected by Seasonal Application Patterns of Trinexapac-Ethyl.” *Crop Science* 40(2):493–97. doi: 10.2135/cropsci2000.402493x.

Fontanier, Charles, Justin Quetone Moss, Lakshmy Gopinath, Carla Goad, Kemin Su, and Yanqi Wu. 2020. “Lipid Composition of Three Bermudagrasses in Response to Chilling Stress.” *Journal of the American Society for Horticultural Science* 145(2):95–103. doi: 10.21273/JASHS04815-19.

Gerlach, Gabriele, Alexander Jueterbock, Philipp Kraemer, Jana Deppermann, and Peter Harmand. 2010. “Calculations of Population Differentiation Based on GST and D: Forget GST but Not All of Statistics!” *Molecular Ecology* 19(18):3845–52. doi: 10.1111/j.1365-294X.2010.04784.x.

Ghimire, M., Boyer, T. A., & Chung, C. (2019). Heterogeneity in urban consumer preferences for turfgrass attributes. *Urban Forestry & Urban Greening*, *38*, 183–192. https://doi.org/10.1016/j.ufug.2018.12.003

Gompert, Zachariah, and Karen E. Mock. 2017. “Detection of Individual Ploidy Levels with Genotyping-by-Sequencing (GBS) Analysis.” *Molecular Ecology Resources* 17(6):1156–67. doi: 10.1111/1755-0998.12657.

Gopinath, Lakshmy, Justin Quetone Moss, and Yanqi Wu. 2021. “Evaluating the Freeze Tolerance of Bermudagrass Genotypes.” *Agrosystems, Geosciences & Environment* 4(2):e20170. doi: 10.1002/agg2.20170.

Goswami, Amit Kumar, Naveen Kumar Maurya, Suneha Goswami, Kirti Bardhan, Sanjay Kumar Singh, Jai Prakash, Satyabrata Pradhan, Amarjeet Kumar, Viswanathan Chinnusamy, Prabhat Kumar, Radha Mohan Sharma, Stuti Sharma, Deepak Singh Bisht, and Chavlesh Kumar. 2022. “Physio-Biochemical and Molecular Stress Regulators and Their Crosstalk for Low-Temperature Stress Responses in Fruit Crops: A Review.” *Frontiers in Plant Science* 13. doi: 10.3389/fpls.2022.1022167.

Gruber, Bernd, Peter J. Unmack, Oliver F. Berry, and Arthur Georges. 2018. “Dartr: An r Package to Facilitate Analysis of SNP Data Generated from Reduced Representation Genome Sequencing.” *Molecular Ecology Resources* 18(3):691–99. doi: https://doi.org/10.1111/1755-0998.12745.

Guo, Yuanwen, Yanqi Wu, Justin Q. Moss, Jeffrey A. Anderson, and Lan Zhu. 2017. “Genetic Variability for Adaptive, Morphological, and Reproductive Traits in Selected Cold-Hardy Germplasm of Common Bermudagrass.” *Crop Science* 57(S1):S-82-S-88. doi: 10.2135/cropsci2016.05.0369.

Hanna, Wayne, Paul Raymer, and Brian Schwartz. 2013. “Warm-Season Grasses: Biology and Breeding.” Pp. 543–90 in *Turfgrass: Biology, Use, and Management*.

Harlan, J. R., J. M. J. de Wet, and K. M. Rawal. 1970. “Geographic Distribution of the Species of Cynodon L. C. Rich (Gramineae).” *East African Agricultural and Forestry Journal* 36(2):220–26. doi: 10.1080/00128325.1970.11662465.

Hellwege, Jacklyn, Jacob Keaton, Ayush Giri, Xiaoyi Gao, Digna R. Velez Edwards, and Todd L. Edwards. 2017. “Population Stratification in Genetic Association Studies.” *Current Protocols in Human Genetics* 95:1.22.1-1.22.23. doi: 10.1002/cphg.48.

Hu, Zhengrong, Jibiao Fan, Yan Xie, Erick Amombo, Ao Liu, Margaret Mukami Gitau, A. B. M. Khaldun, Liang Chen, and Jinmin Fu. 2016. “Comparative Photosynthetic and Metabolic Analyses Reveal Mechanism of Improved Cold Stress Tolerance in Bermudagrass by Exogenous Melatonin.” *Plant Physiology and Biochemistry* 100:94–104. doi: 10.1016/j.plaphy.2016.01.008.

Huang, Bingru. 2008. “Turfgrass Water Requirements and Factors Affecting Water Usage.” *Water Quality and Quantity Issues for Turfgrass in Urban Landscapes. CAST Spec. Publ* 27:193–205.

Huang, Bingru, and Jack D. Fry. 2000. “Turfgrass Evapotranspiration.” *Journal of Crop Production* 2(2):317–33. doi: 10.1300/J144v02n02_14.

Huang, Xuebing, Haiyan Shi, Zhengrong Hu, Ao Liu, Erick Amombo, Liang Chen, and Jinmin Fu. 2017. “ABA Is Involved in Regulation of Cold Stress Response in Bermudagrass.” *Frontiers in Plant Science* 8. doi: 10.3389/fpls.2017.01613.

Hulse-Kemp, A. (2022). *‘Tifway’ reference contig assembly* [Unpublished data]. USDA-Agricultural Research Service.

Karaca, MEHMET, D. J. Lang, G. L. Yerk-Davis, Saha Sukumar, and A. J. Ainsworth. 2000. “Determination of DNA Content and Genome in Cynodon Species by Flow Cytometry.” *Crop Research (Hisar)* 20(1):1–12.

Kassambara, Alboukadel, and Fabian Mundt. 2020. “Extract and Visualize the Results of Multivariate Data Analyses [R Package Factoextra Version 1.0.7].”

Kenna, Michael P. 2008. “Turfgrass and the Environment.” *Water Quality and Quantity Issues for Turfgrasses in Urban Landscapes. CAST Spec. Publ* 27:65–90.

Kilian, Andrzej, Peter Wenzl, Eric Huttner, Jason Carling, Ling Xia, Hélène Blois, Vanessa Caig, Katarzyna Heller-Uszynska, Damian Jaccoud, Colleen Hopper, Malgorzata Aschenbrenner-Kilian, Margaret Evers, Kaiman Peng, Cyril Cayla, Puthick Hok, and Grzegorz Uszynski. 2012. “Diversity Arrays Technology: A Generic Genome Profiling Technology on Open Platforms.” Pp. 67–89 in *Data Production and Analysis in Population Genomics: Methods and Protocols*, *Methods in Molecular Biology*, edited by F. Pompanon and A. Bonin. Totowa, NJ: Humana Press.

Levitt, J. 1980. “Responses of Plants to Environmental Stress, 2nd Edition, Volume 1: Chilling, Freezing, and High Temperature Stresses.” *Responses of Plants to Environmental Stress, 2nd Edition, Volume 1: Chilling, Freezing, and High Temperature Stresses.*

Li, Xin, Quan Cai, Tao Yu, Shujun Li, Sinan Li, Yunlong Li, Yan Sun, Honglei Ren, Jiajia Zhang, Ying Zhao, Jianguo Zhang, and Yuhu Zuo. 2023. “ZmG6PDH1 in Glucose-6-Phosphate Dehydrogenase Family Enhances Cold Stress Tolerance in Maize.” *Frontiers in Plant Science* 14:1116237. doi: 10.3389/fpls.2023.1116237.

Madden, Thomas. 2013. “The BLAST Sequence Analysis Tool.” *The NCBI Handbook* 2:425–36.

Morris, Kevin N., and Robert C. Shearman. 1998. “NTEP Turfgrass Evaluation Guidelines.” Pp. 1–5 in *NTEP turfgrass evaluation workshop, Beltsville, MD*.

Niu, Liangjie, and Wei Wang. 2020. “Defensive Forwards: Stress-Responsive Proteins in Cell Walls of Crop Plants.” 2020.02.15.950535.

Panarella, Michela, and Kelly M. Burkett. 2019. “A Cautionary Note on the Effects of Population Stratification Under an Extreme Phenotype Sampling Design.” *Frontiers in Genetics* 10.

Piepho, H. P., J. Möhring, A. E. Melchinger, and A. Büchse. 2008. “BLUP for Phenotypic Selection in Plant Breeding and Variety Testing.” *Euphytica* 161(1):209–28. doi: 10.1007/s10681-007-9449-8.

Piepho, Hans-Peter, and Jens Möhring. 2006. “Selection in Cultivar Trials—Is It Ignorable?” *Crop Science* 46(1):192–201. doi: 10.2135/cropsci2005.04-0038.

Pina-Martins, Francisco, Diogo N. Silva, Joana Fino, and Octávio S. Paulo. 2017. “Structure_threader: An Improved Method for Automation and Parallelization of Programs Structure, fastStructure and MavericK on Multicore CPU Systems.” *Molecular Ecology Resources* 17(6):e268–74. doi: 10.1111/1755-0998.12702.

Pritchard, Jonathan K., Matthew Stephens, and Peter Donnelly. 2000. “Inference of Population Structure Using Multilocus Genotype Data.” *Genetics* 155(2):945–59. doi: 10.1093/genetics/155.2.945.

Pudzianowska, Marta, and James H. Baird. 2021. “Genetic Diversity and Species-specific DNA Markers of *Cynodon*.” *Crop Science* csc2.20369. doi: 10.1002/csc2.20369.

Purcell, Shaun, Benjamin Neale, Kathe Todd-Brown, Lori Thomas, Manuel A. R. Ferreira, David Bender, Julian Maller, Pamela Sklar, Paul I. W. de Bakker, Mark J. Daly, and Pak C. Sham. 2007. “PLINK: A Tool Set for Whole-Genome Association and Population-Based Linkage Analyses.” *American Journal of Human Genetics* 81(3):559–75.

R Core Team. 2020. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing.

Saitou, N., and M. Nei. 1987. “The Neighbor-Joining Method: A New Method for Reconstructing Phylogenetic Trees.” *Molecular Biology and Evolution* 4(4):406–25. doi: 10.1093/oxfordjournals.molbev.a040454.

Stefaniak, Thomas R., C. A. Rodgers, R. VanDyke, D. W. Williams, and T. D. Phillips. 2009. “The Inheritance of Cold Tolerance and Turf Traits in a Seeded Bermudagrass Population.” *Crop Science* 49(4):1489–95. doi: 10.2135/cropsci2008.09.0564.

Stier, J. C., Steinke, K., Ervin, E. H., Higginson, F. R., & McMaugh, P. E. (2013). Turfgrass Benefits and Issues. In *Turfgrass: Biology, Use, and Management* (pp. 105–145). https://doi.org/10.2134/agronmonogr56.c3

Taliaferro, C. M., A. A. Hopkins, J. C. Henthorn, C. D. Murphy, and R. M. Edwards. 1997. “Use of Flow Cytometry to Estimate Ploidy Level in Cynodon Species.” *International Turfgrass Society Research Journal* 8:385–92.

Taliaferro, Charles M. 2003. “Bermudagrass (Cynodon (L.) Rich).” Pp. 235–56 in *Turfgrass Biology, Genetics, and Breeding*. John Wiley & Sons.

Taliaferro, Charles M., Francis M. Rouquette Jr., and Paul Mislevy. 2004. “Bermudagrass and Stargrass.” Pp. 417–75 in *Warm-Season (C4) Grasses*. John Wiley & Sons, Ltd.

Thomas, Howard, and Helen Ougham. 2014. “The Stay-Green Trait.” *Journal of Experimental Botany* 65(14):3889–3900. doi: 10.1093/jxb/eru037.

Weir, B. S., and C. Clark Cockerham. 1984. “Estimating F-Statistics for the Analysis of Population Structure.” *Evolution* 38(6):1358–70. doi: 10.2307/2408641.

Wickham, Hadley. 2016. *Ggplot2: Elegant Graphics for Data Analysis*. 2nd ed. 2016. Cham: Springer International Publishing : Imprint: Springer.

Xu, Bin, Guohui Yu, Hui Li, Zheni Xie, Wuwu Wen, Jing Zhang, and Bingru Huang. 2019. “Knockdown of STAYGREEN in Perennial Ryegrass (Lolium Perenne L.) Leads to Transcriptomic Alterations Related to Suppressed Leaf Senescence and Improved Forage Quality.” *Plant and Cell Physiology* 60(1):202–12. doi: 10.1093/pcp/pcy203.

Yu, Shuhao, Charles H. Fontanier, Dennis L. Martin, Justin Q. Moss, Carla L. Goad, and Yanqi Wu. 2023. “Effect of Antecedent Drought Stress on Spring Green-up in Turf-Type Bermudagrasses.” *Agrosystems, Geosciences & Environment* 6(2):e20353. doi: 10.1002/agg2.20353.

Yu, Shuhao, Ashley N. Schoonmaker, Liuling Yan, Amanda M. Hulse-Kemp, Charles H. Fontanier, Dennis L. Martin, Justin Q. Moss, and Yanqi Q. Wu. 2022. “Genetic Variability and QTL Mapping of Winter Survivability and Leaf Firing in African Bermudagrass.” *Crop Science* 62(6):2506–22. doi: 10.1002/csc2.20849.

Yue, C., Wang, J., Watkins, E., Bonos, S. A., Nelson, K. C., Murphy, J. A., Meyer, W. A., & Horgan, B. P. (2017). Heterogeneous Consumer Preferences for Turfgrass Attributes in the United States and Canada. *Canadian Journal of Agricultural Economics/Revue Canadienne d’agroeconomie*, *65*(3), 347–383. https://doi.org/10.1111/cjag.12128

Zhang, Xunzhong, E. h. Ervin, C. Waltz, and T. Murphy. 2011. “Metabolic Changes During Cold Acclimation and Deacclimation in Five Bermudagrass Varieties: II. Cytokinin and Abscisic Acid Metabolism.” *Crop Science* 51(2):847–53. doi: 10.2135/cropsci2010.06.0346.

Zhang, Xunzhong, Kehua Wang, E. H. Ervin, C. Waltz, and T. Murphy. 2011. “Metabolic Changes During Cold Acclimation and Deacclimation in Five Bermudagrass Varieties. I. Proline, Total Amino Acid, Protein, and Dehydrin Expression.” *Crop Science* 51(2):838–46. doi: https://doi.org/10.2135/cropsci2010.06.0345.

Zhou, Xiang, and Matthew Stephens. 2012. “Genome-Wide Efficient Mixed-Model Analysis for Association Studies.” *Nature Genetics* 44(7):821–24. doi: 10.1038/ng.2310.

Supplemental Tables

Supplemental Table S3.1 Fertility schedule for all trials included in this study.

		Application Rate (lb N/1000ft^2)			
Application Month	**N-P-K Ratio**	**2017 Trial**	**2018 Nursery**	**2019 Collection**	**2019 NTEP**
May 2017	46-0-0	1.0			
May 2018	46-0-0	1.0			
October 2018	19-0-19	0.5			
March 2019	19-0-19		0.5		
April 2019	19-0-19	1.0		1.0	
May 2019	19-0-19	0.5		0.5	
July 2019	19-0-19	0.5		0.5	0.5
October 2019	21-7-14	1.0	1.0	1.0	
November 2019	21-7-14		0.5		
April 2020	41-0-0	3.0	3.0	3.0	
April 2020	21-7-4				0.5
May 2020	19-0-19				0.5
June 2020	21-7-4				0.5
July 2020	19-0-19			0.5	0.5
September 2020	19-0-19				0.5
October 2020	16-6-8				0.5
November 2020	16-6-8				0.5
December 2020	16-6-8				0.5
February 2021	41-0-0		3.0	3.0	
April 2021	20-0-0		0.5		0.5
June 2021	20.5-0-0	0.5	0.5	0.5	0.5
August 2021	19-19-19	1.0	1.0	1.0	0.5
September 2021	20.5-0-0				0.5
November 2021	20.5-0-0	1.0	1.0	1.0	0.5
April 2022	19-0-9		0.5	0.5	0.5

Supplemental Table S3.2 Descriptive statistics of visual quality (VQ) and color (retention) (VC) across all ratings dates for each study based on all accessions in the study.

Date	Mean	min	max	SD	Mean	min	max	SD
Germplasm Collection		VQ				VC		
January 14, 2021	2.72	1.00	6.00	1.10	2.93	1.00	7.00	1.29
February 2, 2021	3.26	1.00	6.00	1.27	3.05	1.00	7.00	1.43
January 4, 2022	2.22	1.00	5.00	0.88	2.23	1.00	7.00	1.18
2018 Nursery		VQ				VC		
January 11, 2019	4.13	1.00	8.00	0.85	5.20	1.00	9.00	1.76
February 11, 2019	4.87	1.00	7.00	0.94	6.63	1.00	9.00	1.50
February 25, 2019	4.27	1.00	7.00	1.01	5.28	1.00	9.00	1.66
December 31, 2019	4.15	1.00	6.00	0.93	3.30	1.00	8.00	1.37
February 7, 2020	3.28	1.00	6.00	0.98	3.82	1.00	8.00	1.36
December 9, 2020	3.73	1.00	7.00	1.04	3.50	1.00	7.00	0.96
February 3, 2021	3.09	1.00	6.00	0.80	2.84	1.00	7.00	0.98
2017 Trial		VQ				VC		
December 18, 2017	5.98	4.00	8.00	1.19	6.19	4.00	8.00	1.02
December 29, 2017	4.52	2.00	7.00	1.25	5.02	2.00	8.00	1.60
January 19, 2018	4.88	2.00	7.00	1.20	5.46	2.00	8.00	1.70
February 1, 2018	4.96	3.00	7.00	0.94	6.06	3.00	8.00	1.67
February 16, 2018	5.42	3.00	7.00	1.16	6.73	4.00	8.00	1.18
February 26, 2018	3.96	2.00	6.00	0.97	4.58	2.00	7.00	1.41
2019 NTEP		VQ				VC		
December 30, 2019	4.10	3.00	7.00	0.81	5.25	2.00	8.00	1.55
February 6, 2020	4.34	3.00	7.00	1.00	5.96	2.00	8.00	1.53
December 10, 2020	4.63	2.00	7.00	1.26	5.44	2.00	8.00	1.74
January 31, 2021	4.33	2.00	7.00	1.16	4.03	1.00	8.00	1.87
December 13, 2021	3.70	2.00	7.00	0.98	4.50	2.00	8.00	1.57
January 4, 2022	3.04	2.00	7.00	1.05	3.55	1.00	8.00	1.66