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Ploidy Level and Genetic Parameters for Phenotypic Traits in Bermudagrass (*Cynodon* spp.) Germplasm

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Abstract: Bermudagrass (*Cynodon* spp.) is a forage and turf crop commonly used worldwide. The USDA bermudagrass germplasm set is composed of plant introductions (PI's) collected around the world and contains different *Cynodon* species, primarily *C. dactylon*. The collection was screened in a replicated trial in Florida for forage yield, leaf width, nutritive value (NV), and Bermudagrass Stem Maggot (*Atherigona reversura*) (BSM), which is an invasive pest to the southeastern United States that damages bermudagrass fields. The goal of this research was to determine ploidy level and genome size in this USDA collection, and evaluate the influence of ploidy level in the estimation of genetic parameters for BSM, leaf width, dry matter yield, and NV traits. For chromosome counts using classical cytogenetics techniques, root tips and meristems were collected from a set of PI's with known ploidy. The PI's and cultivars with known chromosome counts were used as internal standards to run flow cytometry and estimate genome size of the PI's with unknown ploidy. Ploidy level was determined for all accessions and were used to estimate genetic parameters of phenotypic traits. By providing information on ploidy levels and genetic parameters, this research will support breeding efforts and future selections for forage bermudagrass.

Keywords: *Cynodon*; flow cytometry; polyploidy; bermudagrass stem maggot; forage breeding

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1. Introduction

The common name bermudagrass includes 15 species and two varieties suitable for both forage and turf crops [1]. The genus *Cynodon* comprises monocotyledonous plants that are part of the *Poaceae* grass family. These major forage and turf species are adapted to regions worldwide because of their heat and drought tolerance. *Cynodon* species are widely planted as a warm season crop in semiarid and arid regions [2,3], as well as in the transition zone in the United States [4]. All *Cynodon* species have a basic chromosome number of nine (x = 9) and ploidy levels that range from 2n = 2x = 18 to 2n = 6x = 54 [5,6]. Of the various species, *C. dactylon* (L.) Pers., which contains six varieties, is one of the most genetically variable and economically important species [5,7,8]. The species *C. aethiopicus*, *C. plectostachyus*, and *C. nlemfuensis* are referred to as stargrass, while bermudagrass is commonly used for the other *Cynodon* species [5]. The name bermudagrass will be used thereafter to refer to all genotypes used in this study. Although bermudagrass has origins around the world, many species are native to southern Africa [7] and have evolved into multiple cytotypes [3,9]. While some species have narrow endemics, bermudagrass is distributed around the world between latitudes 45° N and 45° S [10].

Despite their taxonomic differentiation, some *Cynodon* species can cross and produce viable offspring. For instance, *C. nlemfuensis* can outcross with both *C. dactylon* and *C. transvaalensis* [11]. The cultivar "Tifton 85", a widely used forage interspecific hybrid, is a cross between "Tifton 68", a *C. nlemfuensis* hexaploid (2n = 6x = 54) hybrid, and plant introduction (PI) 290884, a tetraploid *C. dactylon* [12]. *Cynodon x magennisii* is a turf plant that came from a naturally occurring cross between *C. dactylon* and *C. transvaalensis* [9]. Advances in breeding techniques have allowed for the development of triploid, pentaploid, and hexaploid hybrid cultivars [13]. For decades, interspecific hybrids between bermudagrass and stargrass have revolutionized the forage industries by providing vital ecosystem services such as forage for livestock, nutrient cycling, maintenance of groundwater quality, and soil erosion control [14–16]. Bermudagrass is grown for hay and pasture in more than 12 million hectares in the southeastern United States [9]. As a turf crop, drought-tolerant cultivars such as "TifTuf" ensure that metabolic functions are maintained during water deficit brought on by irrigation restrictions [17]. Hybrid bermudagrass improves dry matter yield, nutritive value, and turf traits including greater drought tolerance and salt tolerance [18,19].

A large bermudagrass germplasm is maintained at the United States National Plant Germplasm System (USA-ARS NPGS). Many of the accessions in the germplasm are available on the USDA Germplasm Resource Information Network (GRIN) website (https://npgsweb.ars-grin.gov/gringlobal/search/, accessed on 1 April 2021), with *Cynodon dactylon* var *dactylon* containing the majority of the PIs [6]. The accessions consist of both forage and turf cultivars, with forages and uncharacterized populations as the majority. These bermudagrass accessions were collected around the world and include several different species, primarily *C. dactylon*. The germplasm is maintained at the Plant Genetic Resources Conservation Unit in Griffin, GA, and at the USDA Crop Genetics and Breeding Research Unit of the USDA-ARS in Tifton, GA. Given the large number of PIs, a bermudagrass forage core collection was assembled using phenotype evaluations for biotic and abiotic stresses, forage productivity, and cold tolerance [20]. The 169 accessions included in the core collection are composed of PIs and breeding lines from Dr. Glen Burton's former breeding program [20,21]. Large genetic variability is present in this collection [21]; however, this core collection is only a subset of the germplasm that could be exploited for breeding cultivars resilient to abiotic and biotic stresses. Bermudagrass stem maggot (*Atherigona reversura* Villeneuve) (BSM) is an invasive pest that was discovered in the U.S. in 2010 [22] and has since been reported in other parts of the world [23–25]. BSM larva feeding causes damaged tillers and bronzing of fields due to leaf chlorosis [22]. Certain morphological traits in bermudagrass such as tiller density, internode length, and leaf blade width have been found to be correlated to BSM damage [26], but the tolerance and susceptibility to BSM of the accessions in the USDA collection is still unknown.

It is critical to know the ploidy level of the complete list of accessions deposited in germplasm banks. Variation in ploidy level will influence natural mating patterns and gene flow and will determine the viability or sterility of crosses [27,28]. Confirming ploidy level of a cross is also essential if farmers want to produce viable seeds, or if sterile hybrids are desirable. Seeded bermudagrass can serve as an economical alternative and can take less time to establish than vegetative propagation [29]. Sterile interspecific hybrids are a frequent goal for bermudagrass breeding because hybrids cannot contaminate other fields through seed and ensure uniformity when they are planted.

There is large phenotypic variation of morphological and agronomic traits within the *Cynodon* genus, and much of that variation could be the result of multiple ploidy levels in the genus. However, there are limited studies available regarding the influence of ploidy in the estimation of genetic parameters in bermudagrass. In studies of other grass species, ploidy level and DNA content were found to be reliable predictors of phenotypic traits. For instance, a study that used morphological traits to differentiate between diploid and tetraploid annual ryegrass (*Lolium multiflorum* L.) found that traits such as leaf width, plant height, and seed size contained adequate variation to distinguish between the two

ploidy levels [30]. Guard cell length had an especially high correlation (r = 0.87) with genome size and could be used as a cheap and quick indicator of ploidy in annual ryegrass [30]. Recently, Chaves et al. [31] demonstrated that stomata morphometric data can be safely used as an indirect and complementary method for determining the variation in ploidy level in *Cynodon*. In perennial ryegrass (*Lolium perenne* L.), researchers conducted trials to investigate differences with diploid and tetraploid accessions on certain forage traits. They reported that diploid cultivars produced greater herbage accumulation than tetraploids, but tetraploids were more intensively grazed by dairy cows than diploid cultivars [32]. Similarly, an analysis of ploidy level of bermudagrass individuals in China suggests that genetic diversity of *C. dactylon* increased with ploidy level. This was investigated using flow cytometry to determine ploidy level and expressed sequence tag-derived simple sequence repeat loci to estimate genetic diversity [33]. Wu et al. [34] used Chinese bermudagrass accessions to evaluate morphological traits such as establishment rate and weed abundance. They found that when accessions were grouped by ploidy level, tetraploid accessions had greater variability of phenotypic traits compared to pentaploid and hexaploid accessions [34]. The results of these studies indicated that ploidy can be a source for increasing phenotypic diversity in grasses, and it could add a significant effect in the estimation of genetic parameters. Recently, genetic parameters were reported in the USDA bermudagrass collection for the following nutritive value (NV) traits: crude protein (CP), phosphorous concentration (P), in vitro digestible organic matter (IVDOM) and neutral detergent fiber (NDF) [35]. However, the authors did not include the ploidy information in their statistical models.

Here, we aim to: (i) determine and report the ploidy levels of accessions in the USDA National Plant Germplasm System using chromosome counting and flow cytometry, to (ii) phenotypically characterize the germplasm for BSM tolerance, leaf width, dry matter yield (DMY), and NV traits, and to (iii) evaluate the effect of ploidy level in the estimation of genetic parameters for phenotypic traits (NV traits, BSM, DMY, and leaf width) in bermudagrass. A comprehensive list of the ploidy levels and BSM tolerances in the USDA bermudagrass collection and the ability to use this information to select accessions for future crosses will support bermudagrass breeding efforts for the development of high yielding, nutritious, and BSM resistant cultivars.

2. Materials and Methods

2.1. Plant Material

The forage bermudagrass (*Cynodon* spp.) collection for this study was composed of 288 lines, obtained as clonal, vegetative propagules from the USDA-NPGS forage core collection in Tifton, GA and the USDA-NPGS *Cynodon* collection in Griffin, GA. Several cultivars from both collections were included, such as "Florakirk" [36], "Alicia" [19], "Russell" [37], "Mislevy" [38], "Coastal" [39], "Jiggs" [15], Tifton 85 [12], "B2000", "Midland" [40], "Cheyenne" [41], and "Wrangler" [19].

2.2. Experimental Design and Evaluated Traits

A field trial was established at the Plant Science Research and Education Unit, Citra, FL (29°24′16″ N and 82°10′17″ W) in the summer of 2014. The experimental design was a row-column design with two replicates and augmented representation of three controls (Tifton 85, Coastal, and Jiggs). Plants were transplanted from the field to 20 cm wide pots and kept in a greenhouse in Gainesville, FL for the cytological assays.

2.3. Chromosome Counting

Eight accessions were chosen for chromosome counts based on previous ploidy level reports: PI 364484, PI 255450, PI 290884, Tifton 68, PI 316510, Coastal, and Tifton 85 [12,21,39]. There are three candidates for the tetraploid progenitor of Tifton 85, two of which have the same PI number. The germplasm obtained from the USDA had two accessions with the same PI number 290884, which will be addressed as 264 and 251. This

PI was published as the tetraploid progenitor of Tifton 85 in the registration report [12], and was called "Tifton 292" in a new study [42]. Both accessions of PI 290884 were used for chromosome counting.

The chromosome preparations were performed according to the procedures previously described for *Cynodon* by Chiavegatto et al. [43] and Chaves et al. [44]. Briefly, root tips were collected from the accessions potted in the greenhouse and pretreated with 0.0025% cycloheximide (Sigma-Aldrich) for 2 h at room temperature. Subsequently, they were washed in distilled water and fixed in ethanol: acetic acid (3:1) solution overnight. Cell wall digestion was performed with an enzyme mix consisting of 10 µL of pectinase/cellulase solution (100:200 units) (Fisher Scientific), 5 µL of 5% pectolyase (MP Biomedicals), and 5 µL of 5% cytohelicase (Sigma-Aldrich) for 2 h and 15 min at 37 °C. Slides were prepared by the cell dissociation and air drying technique [45].

2.4. Flow Cytometry

The protocol for flow cytometry follows the methodology described by Rios et al. [30] and Anderson et al. [20]. In summary, nucleic extraction buffer and CyStain Propidium Iodide Absolute P staining solution were prepared according to the CyStain® PI Absolute P Kit instructions (Sysmex America, Inc., Lincolnshire, IL, USA), kept in 4 °C, and protected from light. Internal standards included sorghum (*Sorghum bicolor* L.) with a 1C DNA content of 1.74 pg [46], rice (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) with a 1C DNA content of 0.897 pg [47], and the maize inbred line B73 with a genome size of 5.64 pg/2C [48]. Additional bermudagrass standards included diploid (*Cynodon transvaalensis* Burtt Davy) breeding line "AB33", tetraploid (*Cynodon dactylon* (L.) Pers.) cultivar "Celebration", and the PIs with counted chromosomes [49,50]. For each accession, approximately 0.5 cm^2 of healthy, young bermudagrass leaf tissue from a minimum of two separate leaves was collected from the potted plants in the greenhouse and placed in petri dishes. To extract the nucleic DNA, 300 µL of nucleic extraction buffer was pipetted into the petri dishes, the leaves were chopped until completely macerated and an additional 200 µL of extraction buffer was added. The petri dishes were placed on a slant to move the extraction buffer and tissue to the bottom of the plates to sit for an additional 30–60 s. The solution was filtered through a Celltrics® 50 µm filter into 5 mL test tubes. Once the filter was removed, 2 mL of staining solution containing propidium iodide, RNAase, and staining buffer was added to the tubes. The final solution was incubated in the dark at 4 °C for 30–60 min in a closed container with ice.

The BD Accuri™ C6 Flow Cytometer (BD Biosciences, San Jose, CA, USA) was used to obtain 2C DNA content in picograms. The 2C DNA content, measured in picograms, was calculated by multiplying the mean value G1 sample peak by the internal standard DNA content, and dividing the product by the mean value G1 internal standard. The range of 2C DNA content for each ploidy level was based on the 2C DNA content of the accessions with counted chromosomes and previous reports [5,21]. The sample DNA content for the tested accessions were grouped depending on their picogram content and categorized as a certain ploidy level. Table 1 displays the ranges of genome sizes per ploidy level.

2.5. Phenotypic Data Collection

Nutritive value and dry matter yield (DMY) collection was performed as described and reported in Souza et al. [35]. Briefly, plots were harvested every five weeks from April to November of 2015 and 2016. Biomass was collected at 5-cm stubble height from a 1.2 × 3.0 m area in each plot, and dry matter content was estimated using approximately 500 g fresh biomass, and dried in a forced-air oven at 55 °C for 72 h [35]. Concentrations of crude protein (CP), in vitro digestible organic matter (IVDOM), phosphorus (P), and neutral detergent fiber (NDF) were measured by wet chemistry at the University of Florida Forage Evaluation Support Laboratory [35].

Table 1. Range of 2C DNA contents and haploid genome size (1C DNA) in picograms (pg) for accessions in the USDA forage bermudagrass germplasm. Ploidy levels were classified based on their 2C DNA content. The 2C DNA content for accessions with counted chromosome numbers were divided by their ploidy number to obtain their 1C DNA value (pg). The mean 1C DNA values of each ploidy level were averaged, followed by averaging the 1C DNA from all ploidy levels to generate the overall genome size.

Accession	Mean Chromosome Counts ± SE	Ploidy Level	2C DNA (pg)	Average 1C DNA/Ploidy Level
PI 364484	18 ± 2.70	2n = 2x = 18	≤1.59	0.64
PI 255450	27 ± 5.15	2n = 3x = 27	1.60–1.81	0.56
PI 290884, PI 316510, Coastal	36 ± 1.93	2n = 4x = 36	1.82–2.69	0.53
Tifton 85	45 ± 0.82	2n = 5x = 45	2.70–3.25	0.59
Tifton 68	54 ± 0.00	2n = 6x = 54	≥3.30	0.66
			Total Average 1C DNA	0.59

Leaf width measurements were recorded on 30 June 2016. Measurements were taken on five randomly selected leaves per accession. For consistency, the leaves chosen for measurements were located three nodes below the terminal point of the stem and measurements were made on the widest portion of the leaf midsection.

The bermudagrass population was visually rated five times for BSM damage using a scale from 0 to 9 (scale: 0 = no visible damage; 1 = 10% of less damage 9 = more than 90% damage) (Figure 1). The ratings were collected seven times on May, June, August and September 2015, and June, August, and September 2017.

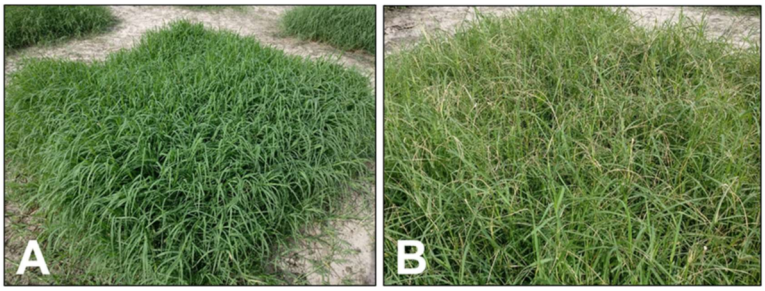

Figure 1. Contrasting bermudagrass response to natural infestations of bermudagrass stem maggot (BSM). The collection was visually rated for BSM tolerance using a 1 through 9 scale (1 = 10 percent or less damage, 9 = 90 percent or more damage). The PI 290664 showed a high tolerance level (**A**), and the cultivar Coastal was one of the most susceptible controls (**B**).

2.6. Statistical Analysis

The estimation of genetic parameters was performed for all traits using a linear mixed model approach using ASReml-R 4.0 (VSN International Ltd, UK) [51], with some variations depending upon data availability. The following model was applied for NV and DMY data:

$$\boldsymbol{y} = \boldsymbol{\mu} + X_1\boldsymbol{\alpha} + X_2\boldsymbol{\beta}_{\boldsymbol{\alpha}} + Z_1\boldsymbol{g} + Z_3\boldsymbol{r}_{\boldsymbol{\beta}} + Z_4\boldsymbol{c}_{\boldsymbol{\beta}} + Z_2\boldsymbol{g}\boldsymbol{\alpha} + \boldsymbol{e}$$

where $\boldsymbol{\mu}$ is the overall population mean; $\boldsymbol{\alpha}$ and $\boldsymbol{\beta}_{\boldsymbol{\alpha}}$ are the fixed effects of harvest and blocks nested to harvest; $\boldsymbol{g}$ is the random effect of entries, with $\boldsymbol{g}$~MVN $(0,\sigma^2_{\boldsymbol{g}}I)$; $\boldsymbol{r}_{\boldsymbol{\beta}}$ and $\boldsymbol{c}_{\boldsymbol{\beta}}$ are the random effects of row and column nested into block, with $\boldsymbol{r}_{\boldsymbol{\beta}}$ ~ MVN$(0,\sigma^2_{r\beta}I)$ and $\boldsymbol{c}_{\boldsymbol{\beta}}$ ~ MVN$(0,\sigma^2_{c\beta}I)$; $\boldsymbol{g}\boldsymbol{\alpha}$ is the random interaction effect between entries and harvest, with $\boldsymbol{g}\boldsymbol{\alpha}$ ~ MVN$(0,\sigma^2_{\boldsymbol{g}\boldsymbol{\alpha}}I)$; and $\boldsymbol{e}$ is the random errors, with $\boldsymbol{e}$ ~ MVN$(0,\sigma^2_{\boldsymbol{e}}I)$. X_1, X_2, Z_1, Z_2, Z_3, and Z_4 are the design matrices associated with their respective vector.

The LW data was analyzed following the same approach, with slight variations in the model. The model for LW data analysis contained just the fixed effects of blocks, and the random effects of row, column, and lines.

For all traits, a second model including the fixed effect of ploidy level was fitted to analyze the impact of the inclusion of the ploidy level information. The ploidy impact was evaluated using the Wald test to check the significance of the ploidy level effect in ASReml-R 4.0. The model excluding the ploidy effect (Base) was compared to a model including ploidy as a fixed effect (Ploidy) considering the following parameters: the Akaike information criteria (AIC), the estimate of broad-sense heritability (H^2), the prediction error variance (PEV), and the selection accuracy. The AIC is based on the decision theory and avoid excessive parameterization; it penalizes models with a large number of parameters. Thus, it is defined by the expression: AIC = -2LogL + $2p$, in which p is the number of model parameters. The lower the value of the AIC, the better the fit of the model.

Broad sense heritability (H^2) was estimated following the ratio: $H^2 = \frac{\sigma_g^2}{\sigma_p^2}$, where σ_g^2 is the genotypic variance, and σ_p^2 is the phenotypic variation. The selection accuracy was estimated using the follow expression: $Acc = \sqrt[2]{(1 - \left(PEV / \sigma_g^2\right))}$, in which PEV is the prediction error variance, and σ_g^2 is the genotypic variance.

A principal component analysis (PCA) was implemented to evaluate the relationship among ploidy levels using the predicted values obtained for seven traits (NDF, CP, P, IVDOM, LW, DMY, and BSM tolerance). The function *prcomp* in R was used for computing the PCA results, and the R package *ggplot2* (Springer-Verlag, New York, NY, USA) [52] was used to visualize the results of the PCA. The phenotypic distribution within each ploidy level was plotted for all traits using the predicted values for each accession and control using the Springer-Verlagpackage *ggplot2* (Springer-Verlag, New York, NY, USA) [52] in R [53].

3. Results

3.1. Chromosome Counting in a Reference Set

In order to have bermudagrass internal standards for flow cytometry, eight accessions from the USDA forage germplasm were used for chromosome counting. We obtained a minimum of ten metaphases with countable chromosomes for each genotype to make a confident count of the number of chromosomes, which, following the basic number of x = 9, ranged from diploid (Figure 2A) through hexaploid (Figure 2F).

Cycloheximide proved to be the most effective pre-treatment for preparing condensed chromosomes based on previous attempts at chromosome visualization using the arresting agents colchicine, monobromonaphthalene, and 8-hydroxyquinoline (data not shown). Of the eight accessions tested, PI 364484 was diploid (2n = 2x = 18), PI 364484 was triploid (2n = 3x = 27), PI 290884 (264 and 251), Coastal, and PI 316510 were tetraploids (2n = 4x = 36), Tifton 85 was pentaploid (2n = 5x = 45), and Tifton 68 was hexaploid (2n = 6x = 54).

3.2. Ploidy and Genome Size Estimation Using Flow Cytometry

The nuclear DNA content of all 288 accessions was successfully determined through flow cytometry (Figure 2). First, the same eight accessions were also assessed by flow cytometry and they became a reference set to determine the thresholds to predict ploidy level in other accessions (Table 1). These thresholds were used to predict the ploidy level and genome size for accessions with no chromosome counts. Flow cytometry output indicated a diversity of ploidy levels ranging from 2× to 6×: 38 were diploid, 63 were triploid, 181 were tetraploid, four were pentaploid, and two were hexaploid (Table S1, Figure 3). Nuclear genome size of the accessions ranged from 0.81 pg to 4.15 pg (Table S1). The average 1C DNA content varied between 0.59 (5×) to 0.64 (2×), and the average 1C genome size across 288 accessions was 0.59 pg (Table 1). Our results indicate that tetraploid is the most common ploidy level of the 288 accessions in the collection.

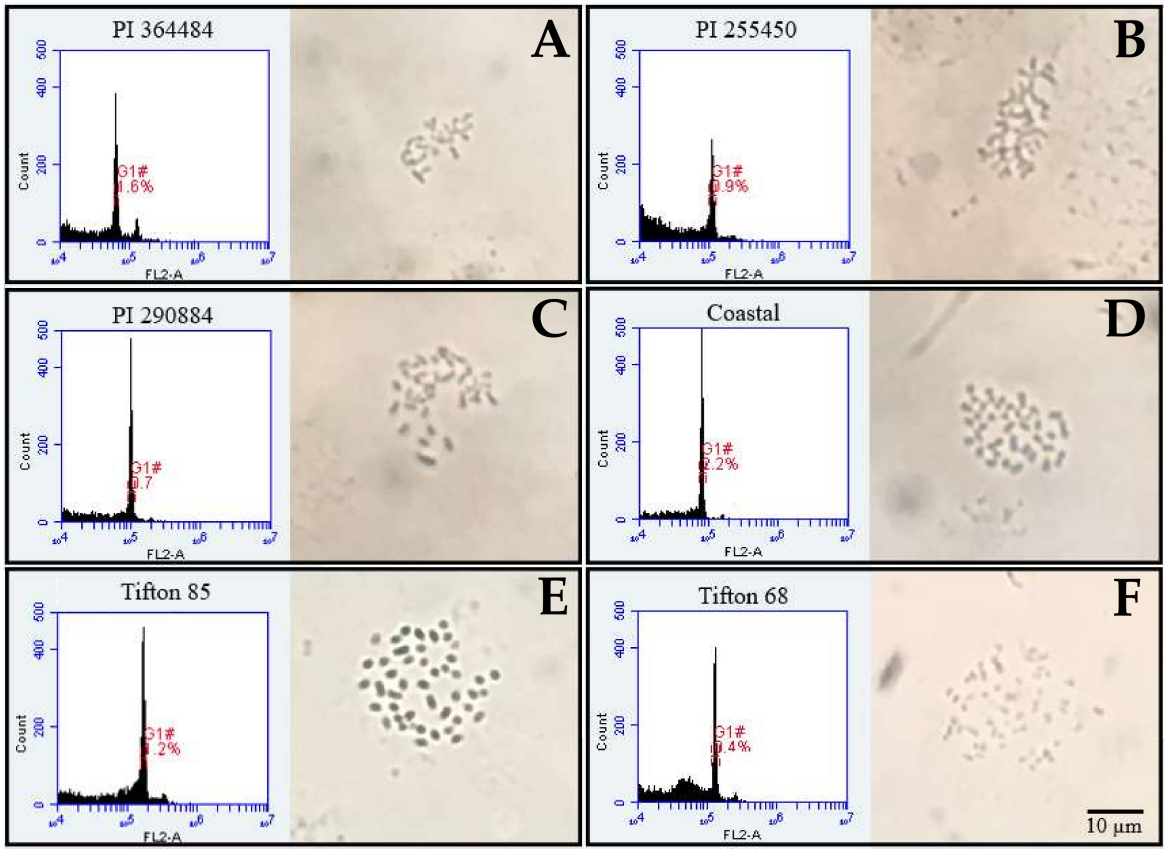

Figure 2. Histograms showing fluorescence intensity of propidium iodide bound to DNA in cell nuclei (**left**) and root tips exhibiting chromosomes under 400x magnification for six bermudagrass genotypes (**right**). Flow cytometry histograms depict the propidium iodide florescence area signals (FL2A) of the sample nucleic DNA in the *x*-axis, and the number of nuclei in the *y*-axis. G1 peaks (red) were gated to indicate the cells in the G1 stage of the cell cycle. The genotypes are showing increasing ploidy levels: (**A**). 2n = 2x = 18, PI 364484; (**B**). 2n = 3x = 27, PI 255450; (**C**). 2n = 4x = 36, PI 290884; (**D**). 2n = 4x = 36, 'Coastal'; (**E**). 2n = 5x = 45, 'Tifton 85'; (**F**). 2n = 6x = 54, 'Tifton 68'. Bar = 10 µm.

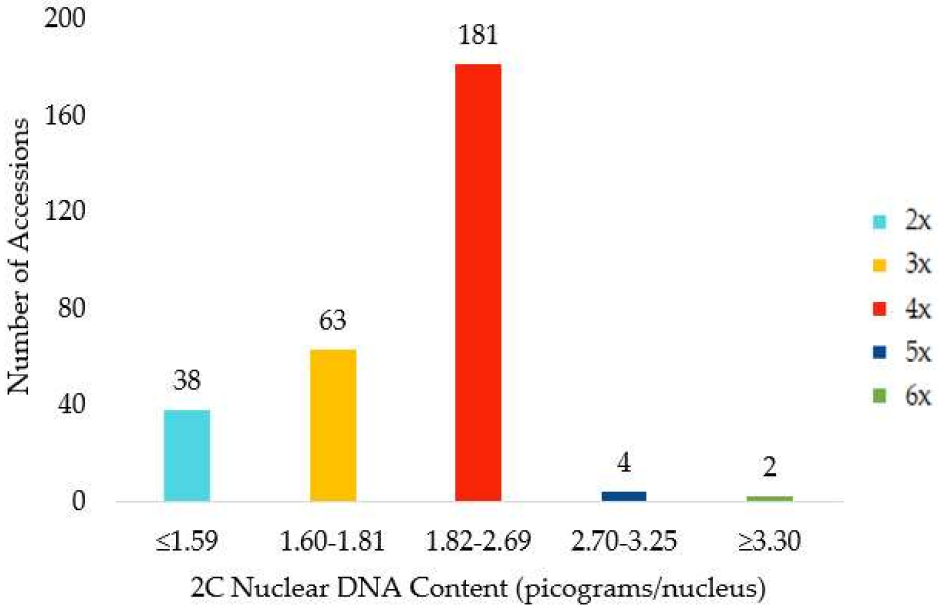

Figure 3. Nuclear DNA content (pg) of 288 accessions of bermudagrass from the USDA collection determined through flow cytometry.

3.3. The Effect of Ploidy in the Estimation of Genetic Parameters

The relationship between ploidy level and phenotypic traits was studied by estimating variance components and genetic parameters using linear mixed models. We excluded the two 6x accessions due to lack of representation at this ploidy level. Ploidy level had a significant effect ($p < 0.05$) for LW, BSM, DMY, IVDOM, and NDF (Table 2), however, the inclusion of ploidy as a factor led to small improvements in the model fitness (Table 3). For instance, the AIC was lower for the model that included ploidy for LW, IVDOM, BSM, DMY, and NDF. Nevertheless, the model with ploidy did not increase the H^2 estimate for any trait, when compared to the base model. Instead, H^2 was lower when ploidy was included for LW, BSM, IVDOM, DMY, and NDF, while it was equal for CP, and P. The highest H^2 was estimated for LW (0.83) without including ploidy in the model, and it decreased to 0.81 for the ploidy model (Table 3). The coefficient of variation (CV) did not change between models (±0.05) for LW, DMY, and CP, while it was lower for BSM, IVDOM, and NDF for the models including ploidy (Table 3). Additionally, the significant differences in ploidy level for LW, BSM tolerance, IVDOM, DMY, and NDF did not improve the accuracy of the models (Table 3).

Table 2. Mean values and standard error (SE) of each ploidy level for leaf width (LW, cm), Bermudagrass Stem Maggot (BSM) tolerance (1 = 10 percent or less damage, 9 = 90 percent or more damage), nutritive value traits (g kg^{-1}): crude protein (CP), phosphorus (P), in vitro digestible organic matter (IVDOM), neutral detergent fiber (NDF), and dry matter yield (kg ha^{-1}) (DMY) based on predicted values of all the USDA forage bermudagrass germplasm accessions for each trait.

	LW		BSM		CP		P		IVDOM		NDF		DMY	
Ploidy	**Mean**	**SE**	**Mean**	**SE**	**Predicted**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Predicted**	**SE**	**Predicted**	**SE**
2	3.01 b	0.18	2.57 b	0.16	135.1 a	0.25	3.0 a	0.01	463.8 b	0.84	680.4 b	0.39	1726.99 b	95.90
3	2.41 c	0.14	3.03 ab	0.13	132.6 a	0.21	2.8 a	0.01	445.5 b	0.66	697.1 a	0.31	1797.46 b	75.50
4	2.64 bc	0.08	3.18 a	0.08	132.2 a	0.14	2.9 a	0.01	449.1 b	0.41	695.5 a	0.19	1898.81 b	45.80
5	4.39 a	0.47	3.09 ab	0.47	139.5 a	0.75	3.1 a	0.02	558.0 a	2.50	674.3 b	1.15	2994.83 a	292.20

Means within traits followed by the same letter are not significantly different at $p < 0.05$ according to Tukey's Honest Significant Differences Mean Separation test.

Table 3. Comparison of models excluding (Base) and including ploidy as a fixed effect for the estimation of broad-sense heritability (H^2), Coefficient of Variation (CV), Akaike Information Criteria (AIC), and accuracy for the USDA forage bermudagrass germplasm for the following traits: leaf width (cm) (LW), Bermudagrass Stem Maggot (BSM) tolerance (1 = 10 percent or less damage, 9 = 90 percent or more damage), dry matter yield (g kg^{-1}) (DMY), and the following nutritive value traits expressed in g kg^{-1}: crude protein (CP), phosphorus (P), in vitro digestible organic matter (IVDOM), neutral detergent fiber (NDF).

	H^2		CV		AIC		Accuracy	
Trait	**Base**	**Ploidy**	**Base**	**Ploidy**	**Base**	**Ploidy**	**Base**	**Ploidy**
LW	0.83	0.81	17.13	17.18	397	386	0.89	0.87
BSM	0.47	0.46	34.57	30.42	1181	1177	82.83	80.73
CP	0.32	0.32	8.00	7.99	2395	2404	51.1	49.42
P	0.52	0.51	13.67	15.37	−5544	−5522	75.87	74.19
IVDOM	0.40	0.38	37.61	34.18	4723	4701	66.65	61.80
NDF	0.53	0.51	6.44	6.07	2964	2952	75.36	72.88
DMY	0.44	0.42	8.5	8.5	97,541	97,494	96.2	94.9

Despite the lack of model improvement to estimate variance components and calculate genetic parameters, trends were observed for mean values across ploidy levels. Mean values for LW were similar for 2× (3.01 cm), 3× (2.41 cm) and 4× (2.64 cm), but 5× showed significantly greater LW with a mean of 4.39 cm. There was a wide range of variation in LW for 2x accessions, and to a lesser extent for 3× and 4×, while pentaploid

accessions had the widest leaves and smallest range of variation for LW (Figure 4A). Similar trends were observed for IVDOM and DMY, 5× accessions had greater means, but the other ploidy levels showed greater variation (Table 2). The ploidy level effect was less evident when comparing mean values for BSM and NDF. The means for BSM tolerance were significantly different between 2× (2.57) and 4× (3.18) accessions, but not between 3× (3.03) and 5× (3.09). Meanwhile, the means for NDF were not significantly different between 2x and 5×, or 3× and 4×, but they were significantly different between those two groups. There were no significant differences among ploidy levels for CP or P (Table 2). Our results suggest that each phenotypic trait had a different response to the addition of ploidy in the model.

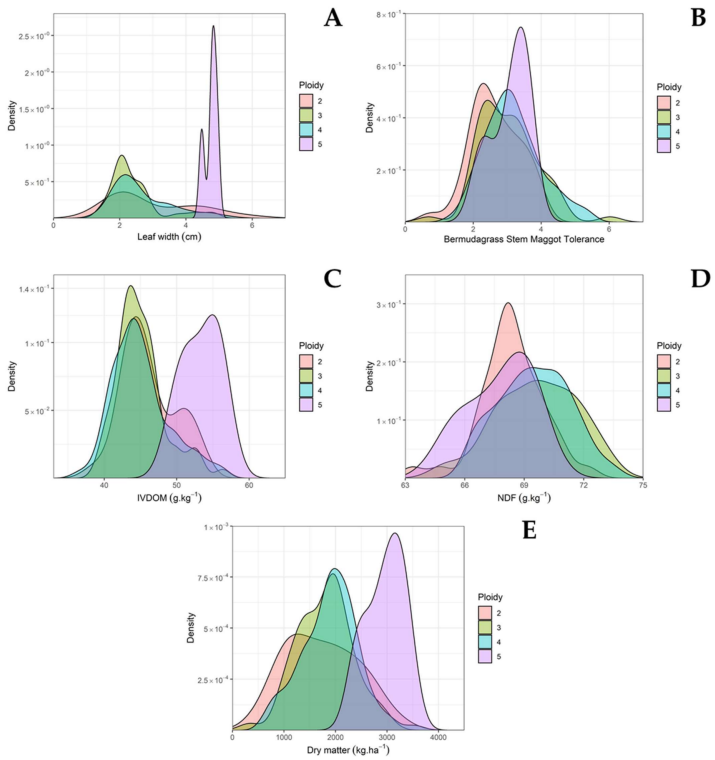

Figure 4. Density plots showing the phenotypic trait variation by ploidy level for (**A**) leaf width (cm), (**B**) Bermudagrass Stem Maggot tolerance, (**C**) in vitro digestible organic matter (IVDOM), (**D**) neutral detergent fiber (NDF), and (**E**) dry matter yield. Traits were measured in accessions of bermudagrass from the USDA collection.

3.4. Phenotypic Diversity among Accessions and Cultivars

Significant differences among ploidy levels were observed for LW, BSM tolerance, DMY, IVDOM, and NDF (Table 2), which were reflected in the broad phenotypic range for all traits (Figure 4). The genetic variation was significant for all traits in all models based on the likelihood ratio test (data not shown). Additionally, the accessions presented phenotypic values more extreme than any cultivar across all traits (Figure 5A–E). For leaf width, a great number of accessions had narrower leaf blades than the population mean and Florida 44 (control with the narrowest leaf blades), while thirteen accessions had wider leaves than Tifton 85 (Table S1). The PI 290895 had the narrowest leaves (1.17 cm), while PI 224566 had the widest leaves (6.12 cm). Among cultivars, Coastal had a leaf width similar to the population mean and Tifton 85 had wider leaves (Figure 5A). A wide range of variation was observed for BSM in this population. The most tolerant accession was PI 606545 (average rating of 0.69), and the most susceptible was PI 287246 with a rating of 6.03. The average BSM rating across all accessions was 3.06, and Coastal was the most susceptible cultivar, while Tifton 85 was the most tolerant cultivar (Figure 5B). Crude protein showed a wide range for accessions. While all controls had similar mean CP to

the population mean (Figure 5C), the PI 292601 showed the highest CP (165.4 g kg^{-1}), and PI 364490 showed the lowest value (112.4 g kg^{-1}). Phosphorus content of Tifton 85 and the population mean were almost identical, while the other cultivars were more dispersed around the mean (Figure 5D). The accession with the highest P content was PI 316507 (4.1 g kg^{-1}) while the accession with the lowest content was PI 291148 (2.2 g kg^{-1}). Most of the IVDOM values for the cultivars were above the population mean, except for Coastal (Figure 5E). The highest IVDOM value was by the cultivar Tifton 84 (567.7 g kg^{-1}) and the lowest value was from PI 291155 (360.4 g kg^{-1}). All the cultivars had lower NDF values than the population mean except for Tifton 85 (Figure 5F). The PI 291148 had the highest NDF (737.1 g kg^{-1}) and PI 297827 had the lowest (633.6 g kg^{-1}). Finally, all the cultivars had higher DMY values than the population mean, with Tifton 85 exhibiting the highest DMY of the cultivars included as checks (Figure 5F). PI 316510 had the highest DMY of 3617.67 kg ha^{-1} and PI 606545 had the lowest DMY of 328.20 kg ha^{-1}.

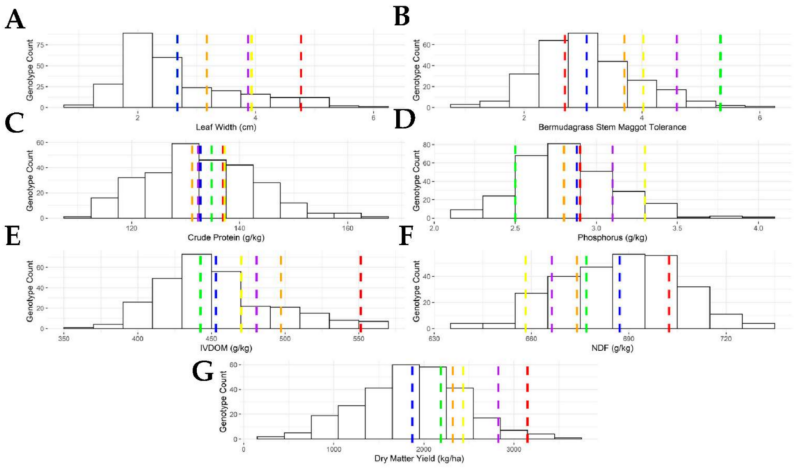

Figure 5. Histogram for five traits using predictive values estimated for the USDA-NPGS bermudagrass germplasm collection and five checks: Tifton 85 (red), Mislevy (green), Florida 44 (orange), Jiggs (purple), and Coastal (yellow). Traits: (**A**) leaf width (cm), (**B**) Bermudagrass Stem Maggot tolerance (1 = 10 percent or less damage, 9 = 90 percent or more damage), and the following nutritive value traits expressed in g kg^{-1}: (**C**) crude protein, (**D**) in vitro organic matter digestibility (IVDOM), (**E**) nutrient detergent fiber (NDF), and (**F**) phosphorus, and (**G**) dry matter yield (kg ha^{-1}). The dashed colored lines represent the means for each check, and the blue lines represents the population mean.

A principal component analysis (PCA) was run to examine the structure of covariation within the germplasm, their ploidy, and phenotypic performance for all traits (Figure 6). The first principal component (PC) explained 39.13% of the variability in the data set, with NV and LW traits providing the higher variation. The second PC explained 24.42%, and DMY had the higher contribution in the variation. The PCA results reaffirm the lack of association between ploidy levels and the phenotypic traits, and also showed the higher association of the pentaploid lines with the LW and IVDOM. The trait correlations for LW and IVDOM were highly positively correlated while both variables were moderate for DMY. The traits CP and P were also highly correlated, and both traits were negatively correlated with NDF (Figure 6). Overall, the accessions showed greater phenotypic diversity than the cultivars (Figure 6). Tifton 85 showed greater DMY, LW and IVDOM. FL44 and Jiggs had higher LW and IVDOM, and Jiggs also had higher P (Figure S1). Although there was no direct grouping by ploidy, the majority of the pentaploid accessions showed greater DMY, LW, and IVDOM. We identified a group of accessions, consisting of multiple ploidy levels, that had greater LW, IVDOM, and higher DMY. On the contrary, another cluster of 2×, 3×, and 4× accessions showed lower DMY and lower NDF, but had greater CP and P. There was a large cluster of accessions with high NDF values but low CP and P. The BSM had a very low contribution to explaining the variation in PC1 and PC2 for this germplasm.

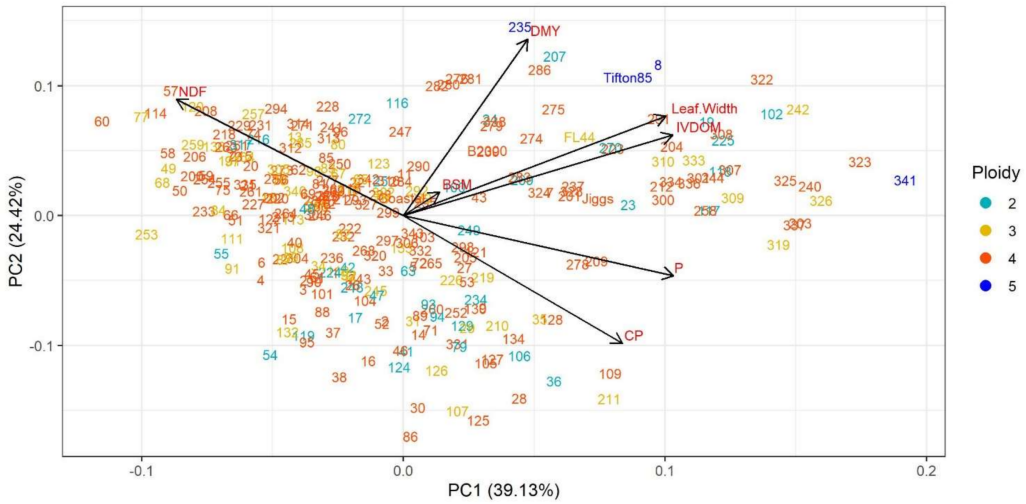

Figure 6. Principal Component Analysis (PCA) of ploidy level and Leaf Width (cm), Bermudagrass Stem Maggot tolerance (1 = 10 percent or less damage, 9 = 90 percent or more damage), Crude Protein (g kg^{-1}), Phosphorus concentration (g kg^{-1}), In Vitro Digestible Organic Matter (g kg^{-1}), Nutrient Detergent Fiber (g kg^{-1}), and Dry Matter Yield (g kg^{-1}). Colors denote ploidy levels of each accessions, included as their designated entry numbers.

4. Discussion

Food production must increase 70% by 2050, including 200 M more tons of beef and other livestock products [54]. The United States is the world's largest beef producer, the second largest beef exporter, and milk has a farm value of production second only to beef among livestock industries [55]. Feed represents the highest single-factor production cost and typically accounts for 40–60% of livestock production costs [56]. Therefore, utilizing pastures as a feed source is re-gaining interest primarily because of economic motivations, followed by environmental, and social interests. The southeastern United States and other subtropical regions worldwide offer unique agroecosystems to grow high-yielding and nutritious forage for grazing and hay. Bermudagrass is the most used warm-season perennial forage species grown in 12 million hectares in the southeastern United States [9]. We determined the ploidy level and genome size for a large and diverse bermudagrass germplasm collection from the USDA, and then evaluated the effect of ploidy level on the estimation of genetic parameters for critical phenotypic traits. The results obtained in this study will aid plant breeders in the development of new bermudagrass cultivars with improved DMY, NV, and tolerance to BSM.

4.1. Ploidy Level and Genome Size Estimation Using Flow Cytometry

The protocol used to visualize C-metaphases produced suitable chromosomal preparations with condensed and spread chromosomes. In general, polyploid species are known to have smaller chromosomes [57]. Warm-season grasses tend to have small chromosomes, specifically *Cynodon* somatic chromosomes, which makes chromosome counting more difficult [58]. Interestingly, warm-season grasses tend to have more chromosomes in a given ploidy level compared to cool-season grasses, yet cool-season grasses have a larger genome size. Arumuganathan et al. [59] reported that diploid African bermudagrass line DTC 95 had 18 chromosomes and a 2C DNA content of 1.03 pg, while cool-season, diploid perennial ryegrass cultivar Brightstar only had 14 chromosomes but has a higher 2C DNA content of 5.66 pg [59]. The larger genome size of Brightstar can be attributed to the cool-season cultivar having larger chromosomes.

The flow cytometry analysis revealed that the range in genome size for 4× is much larger than the other ploidy levels in this bermudagrass collection. This could be attributed to the widespread variation in 4× *Cynodon* plants around the world. Considering that there are over five tetraploid bermudagrass taxa that can readily hybridize, including *C. dactylon*

var. *dactylon* and *C. arcuatus* [9], it can be inferred that tetraploid accessions contain wider diversity because of the cross compatibility of taxa with various origins and phenotypes. For example, *C. dactylon* var. *coursii* plants are tall and nonrhizomatous, while *C. dactylon* var *dactylon* plants are rhizomatous and have a variety of sizes depending on their origin [9]. Although these varieties are tetraploid, they widely vary in morphology and genome size. Besides, according to Zhang et al. [60] polyploidization may alter the DNA 1C values of bermudagrass individuals if it is coupled with adaptation to different environments. Varying climatic conditions at different latitudes might favor plants possessing certain genome sizes and ploidy levels if they are able to tolerate stressful environments. Considering that there are more tetraploid bermudagrass genotypes found in nature [2,20,60,61], there was a high probability of the tetraploid cytotype possessing a wider range of 2C DNA content than the other ploidy levels as observed by Chaves et al. [44]. Because of the expected higher range in genome size at the 4× level, it was necessary to count chromosomes of multiple 4× accessions to obtain accurate estimates for 1C DNA values (Table 1). Using chromosome counts to identify four tetraploid accessions provided us with additional tetraploid standards for flow cytometry to confirm that tetraploid forage bermudagrass has a wider range than the other ploidy levels.

The average genome size (pg/2C DNA) for each ploidy level determined in this study was similar to previous reports [5,34,43,59,60,62,63], indicating that our genome size estimations are consistent with studies in bermudagrass (Table 4). The slight differences in average genome size per ploidy level in this study and previous reports was expected due to the use of different species and germplasm within species, and the use of external standards for genome size measurement. Best practice methods of genome size estimation emphasize the use internal standardization [64] as in the current study. For instance, Wu et al. [65] evaluated 132 accessions of Chinese bermudagrass, but they only used *C. dactylon*. Arumuganathan et al. [59] reported the 2C DNA content and ploidy level from four bermudagrass cultivars and selections, which were different species and interspecific hybrids. They included the cultivar "Savannah" which is a *Cynodon dactylon* (L.) Pers., an African bermudagrass of *Cynodon transvaalensis* Burtt-Davy, and the two turf hybrid cultivars "Tifgreen" and "Tifway," which are crosses of *Cynodon dactylon* x *C. transvaalensis* and *Cynodon transvaalensis* x *C. dactylon*, respectively. Taliaferro et al. [5] evaluated USDA ARS and Oklahoma State University accessions from various species, including triploid crosses of *C. dactylon and C. transvaalensis* and 14 hexaploidy crosses of *C. dactylon and C. transvaalensis*. Pang et al. [63] evaluated accessions from the University of Florida turfgrass germplasm collection from a variety of *Cynodon* species [63]. More recently, Chiavegatto et al. [43] provided the nuclear DNA content and ploidy level of seven accessions from the USDA bermudagrass germplasm in Tifton, GA. The authors used multiple *Cynodon* species including *C. nlemfuensis* Vanderryst, *C. transvaalensis* Burtt-Davy, and *C. dactylon* var. *polevansii*. Finally, Zhang et al. [60] evaluated patterns in ploidy level and genome size of 216 accessions of *C. dactylon* by flow cytometry from 16 geographic sites across of China and obtained the 2C DNA content for each bermudagrass ploidy level.

Table 4. Comparison of average genome size (pg/2C DNA) per ploidy level for six different studies including bermudagrass accessions.

	2C DNA Content (pg)						
Ploidy Level	**Grossman et al. (This Study)**	**Wu et al. 2006 [65]**	**Taliaferro et al. 1997 [5]**	**Pang et al. 2010 [63]**	**Chiavegatto et al. 2016 [43]**	**Arumuganathan et al. 1999 [59]**	**Zhang et al. 2020 [60]**
2x	1.26	1.6	1.11	1.17	1.08–1.17	1.03	2.38
3x	1.69	2.18	1.6	2.19	1.63	1.61; 1.37	2.41
4x	2.11	3.13	2.25	3.06	1.88–2.10	1.95	2.43
5x	2.96			2.47	2.55		2.87
6x	3.94	4.105	2.8	4.02			3.28

The main factor related to the genome size variation could be the rearrangements with losses or gains of sequences, resulting from the processes of hybridization and polyploidy [66,67]. Moreover, differences in heterochromatic composition (repetitive sequences) can also affect the amount of nuclear DNA, which was pointed out in accessions of *C. dactylon* that showed variability in the number and size of CMA^+ and $DAPI^+$ bands [44]. Future analyses determining ploidy levels of bermudagrass accessions using flow cytometry can use our reported 2C DNA range as a reference and to create an amended range by running accessions with confirmed ploidy level from chromosome counts. Since technical variation due to using different flow cytometers can exists, minor variations in sample DNA content (pg/2C) calculations can lead to incorrect ploidy level estimation.

The two entries labeled as PI 290884 (264 and 251) and Tifton 282 were all classified as tetraploid (2n = 4x= 36; Table S1), and Tifton 68 was confirmed as hexaploid (2n = 6x = 54; Table S1). Burton et al. [12] used the tetraploid *C. dactylon* PI 290884 and crossed it with hexaploid *C. nlemfluensis* Tifton 68 to create Tifton 85, one of the most productive forage bermudagrass cultivars. Our results confirmed that Tifton 85 is pentaploid (2n = 5x = 45), and other pentaploid entries are available in this collection (Table S1).

4.2. Evaluated Traits

The inclusion of ploidy information as a covariate in the mixed models did not provide a significant improvement in the estimation of variance components and the calculation of genetic parameters. Nevertheless, the statistical significance of genotypic variance for all traits shows evidence for a genetic control of these traits. Based on the moderate H^2 exhibited by the DMY, NV, and BSM traits in this collection, the genetic parameters analyzed in this study indicated that genetic gains are anticipated for the traits. The H^2 explains the magnitude of phenotypic variance due to the genetic variance, and all traits showed moderate to high estimates. In particular, the high H^2 for LW and significant differences among ploidy levels means that LW would be an ideal trait for breeding purposes. The moderate H^2 for BSM, DMY, and the NV traits indicates that the expression of these traits are more impacted by the environmental component, and the selection for these traits would require more phenotyping efforts.

Information about ploidy level is critical for breeding purposes, as some traits showed a wide range of phenotypic variation within ploidy levels. Depending on the final use for forage bermudagrass cultivars, the selection for fine or coarse leaves would be preferred. For green-chop and grazing systems, cultivars and breeding lines exhibiting wider leaves, such as Tifton 85, would be ideal, also because they produced higher yields and IVDOM (Figure 6). Alternatively, finer leaves and stems are favored when breeding for bermudagrass for hay systems because they dry faster. Many 2×, 3× and 4× accessions had finer leaves than the available cultivars (Figure 5A). Beyond their use for hay, narrow-leaved cultivars are also preferred for feeding horses [68]. It has also been hypothesized that LW can also affect BSM tolerance. This was supported by data that reveals thinner-stemmed varieties like "Common" and "Alicia" possess higher rates of BSM insect feeding [69]. Additionally, a Japanese study reported that although BSM damage has a positive relationship with tiller diameter, it has a negative correlation to leaf blade width [22,26]. Investigating a correlation between LW and BSM would be beneficial because it would encourage growers to select cultivars with certain leaf widths to increase BSM tolerance in densely afflicted fields. In our study, LW and BSM did not show a correlation, and we found susceptible accessions with fine and coarse leaves. LW might not have been significantly different between diploid and tetraploid bermudagrass, but it was significantly different for annual ryegrass, in which tetraploid plants had wider leaf blades than diploid plants [30]. In fact, diploid and tetraploid ryegrass were also significantly different for biomass [30], while DMY was only significant for pentaploid accessions for bermudagrass (Table 2). Knowing which ploidy levels contain the highest DMY is essential because high yields are always desired by forage producers.

The results presented in this study revealed that there is not a single accession resistant to BSM in this germplasm. However, several lines were identified as highly tolerant and could serve as potential sources for breeding BSM-resistant bermudagrass in the future. For instance, diploid accessions would be a good source for resistance to BSM since they had overall lower damage (Table 3). Besides, chromosome doubling can be performed on those 2× accessions to create 4×, which will then be used to breed bermudagrass hybrids. We did not find correlations between BSM and other traits in the 288 accessions, so the mechanism for tolerance would need to be further explored in future studies. Baxter et al. [69] found that finer-leaved cultivars were more susceptible to BSM damage, and Tifton 85 was the most tolerant cultivar. Our results confirmed previous findings with cultivars, but it did not extend to the whole collection. Availability of BSM tolerance ratings and knowledge of the BSM variation within the collection will benefit breeders developing improved bermudagrass forage cultivars and ultimately growers to aid in selecting cultivars that will withstand BSM intrusions with minimal damage.

For CP and P, ploidy was not significant, so there is no need to consider ploidy when selecting accessions with high CP and P. This result was congruent with the findings by Balocchi and Lopez [32], who found CP to have no significant differences between diploid and tetraploid perennial ryegrass. However, they did find the two ploidy levels to have significant differences for IVDOM, while our diploid and tetraploid bermudagrass accessions were not significantly different for IVDOM (Table 2). The non-significance of CP could be related to the availability of nitrogen in the soil. A recent study of nitrogen use efficiency found that applying nitrogen significantly increased crude protein contents of *C. dactylon* [70]. This study used 283 of the bermudagrass accessions from the USDA germplasm and included ploidy levels ranging from 2×–6×. They found that many of the accessions with high nitrogen use efficiency changed their protein content depending on the nitrogen rates [70]. This information tells us that a reason ploidy level did not influence CP content might be because the nitrogen application had a stronger impact on CP content than ploidy.

The significant difference in pentaploid accessions for IVDOM and DMY shows the impact that pentaploid interspecific hybrids can have on improving forage bermudagrass cultivars. As previously noted, Tifton 85 possesses a superior phenotype to the other cultivars and accessions, as it performed better than all cultivars for BSM tolerance, CP, IVDOM, NDF, and DMY (Figure 5A–G). Additionally, the sterile F_1 hybrid Florakirk, released in 1994, was confirmed to be a pentaploid through flow cytometry. This cultivar showed very similar LW and BSM rates to Tifton 85 in our study (Table S1). Yet, when the two cultivars were grown in northern Florida, Tifton 85 produced superior herbage accumulation, IVDOM, and persistence [71]. Finally, Breeding Line 235, another confirmed pentaploid, has wider leaves (Table S1) and higher DMY than Tifton 85 (Figure 5). For this reason, Breeding Line 235 would be an ideal accession to explore for cultivar release in the near future. Similar analysis can be done with the data presented in this study to evaluate sterile and fertile accessions for release or crosses, respectively.

The wide phenotypic variation within ploidy level observed in all traits (Figure 4A) may be due to the presence of multiple species, interspecific hybrids, and diversity within the same species for any given ploidy level. To illustrate, *C. dactylon* has multiple varieties that are diploid and tetraploid, yet they all differ in phenotype [7]. Meanwhile, both the species *C. dactylon* and *C. nlemfuensis* contain tetraploid genotypes, even though they originated from different parts of the world and are thus phenotypically variable [9]. These intra- and interspecies variations in bermudagrass could also be part of the reason ploidy did not improve the models in any of the reported traits. A future study to follow this research could include the use of molecular markers to investigate the genetic diversity and identify the species diversity in each bermudagrass ploidy level.

The ploidy level information and the phenotypic performance for each ploidy level (Table 2) and for each accession (Table S1) will benefit geneticists and breeders when selecting lines for crosses. For BSM tolerance, all the accessions to the left of the mean

(blue) and the check, Tifton 85 (red) have low damage, and thus a higher tolerance to BSM (Figure 5B). Similarly, all the cultivars and many of the accessions have yields higher than the population mean (Figure 5G). This data opens up the ability to use these accessions for crosses to develop high-yielding and more nutritious bermudagrass cultivars. For example, the PI 316510 showed high yield, high nutritive value and BSM tolerance among accessions, and it was released as a cultivar named "Newell" by the University of Florida (Dr. Esteban Rios, personal communication). Other accessions are currently being evaluated in multi-location trials in the southeastern United States and will be considered for releases in the future.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11050912/s1, Figure S1: Principal Component Analysis (PCA) of ploidy level and Leaf Width (cm), Bermudagrass Stem Maggot tolerance (1 = 10 percent or less damage, 9 = 90 percent or more damage), Crude Protein (g kg^{-1}), Phosphorus concentration (g kg^{-1}), In Vitro Digestible Organic Matter (g kg^{-1}), Nutrient Detergent Fiber (g kg^{-1}), and Dry Matter Yield (g kg^{-1}). Cultivars are in light blue and accessions, written as their entry numbers, are in dark blue., Table S1: Ploidy level, 2C DNA content in picograms (pg), origin, Bermudagrass Stem Maggot (BSM) tolerance (1 = 10 percent or less damage, 9 = 90 percent or more damage), and leaf width (cm) of 287 bermudagrass accessions from the USDA germplasm collection.

Author Contributions: E.F.R. conceived the project. E.F.R. and Y.L. collected the phenotypic data in Florida. A.Y.G., A.L.A.C., M.T.M.F. and V.H.T. counted chromosomes. A.Y.G., M.H.M.L.A. and E.F.R. analysed the phenotypic data. A.Y.G. and E.F.R. wrote the manuscript. K.B., K.E.K. and E.F.R. supervised A.Y.G. and reviewed the first draft. All authors have read and agreed to the published version of the manuscript.

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