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Natural Genetic Diversity of Nutritive Value Traits in the Genus *Cynodon*

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Abstract: The *Cynodon* spp. collection maintained by United States Department of Agriculture National Plant Germplasm System (USDA-NPGS) has limited information on nutritive value (NV) traits. In this study, crude protein (CP), phosphorous concentration (P), in vitro digestible organic matter (IVDOM), and neutral detergent fiber (NDF) were determined to (i) estimate genetic parameters for NV, (ii) obtain genetic values for the whole population across two harvests, (iii) estimate genotype by harvest interaction (GHI) for NV traits, and (iv) select accessions exhibiting improved NV traits compared to 'Tifton 85'. The experiment was setup as a row-column design with two replicates and augmented representation of controls: Tifton 85, 'Jiggs', and 'Coastal'. The whole-population was harvested twice, and data were analyzed using linear mixed models with repeated measures. In addition, a selected population of 15 genotypes were evaluated across 11 harvests to determine the extent of GHI. Genetic parameters revealed the presence of significant genetic variability, indicating potential improvements for NV through breeding. Specifically, P and IVDOM presented large variation, while NDF had lower diversity but some accessions exhibited lower NDF than Tifton 85. Low GHI, except for IVDOM, indicated genotypic stability and potential for selecting improved accessions under fewer harvests. Breeding line 240, PI-316510, and PI-3166536 presented superior NV than Tifton 85.

Keywords: bermudagrass; forage breeding; genetic parameters; genotype by harvest interaction; Tifton 85

1. Introduction

The investigation of natural genetic diversity present in germplasm banks is a key step to improve traits with narrow genetic variability. The use of germplasm banks is even more important for perennial forages, as several important releases worldwide originated from selections of plant introductions made in large collections [1]. Bermudagrass (*Cynodon* spp.) is the most widely used warm-season perennial forage for hay and pasture in the southeastern United States, covering more than 12 million hectares [2]. Its popularity among livestock and hay producers lays on high biomass production, nutritive value, animal performance, fast-curing for hay production, and drought tolerance [3–7]. The genus *Cynodon* is composed of genetically diverse species of variable ploidy level [8,9]. The most agronomically valuable species are *Cynodon dactylon* Pers. and *Cynodon nlemfuensis* Vanderyst. Both of

these are cross-compatible, and several improved interspecific hybrids have been developed, selected, and released commercially: Coastcross I, Tifton 85, Florakirk [10–14].

The collection of United States Department of Agriculture National Plant Germplasm Systems (USDA-NPGS) for *Cynodon* is maintained by the Agricultural Research Service Coastal Plain Experimental Station in Tifton, GA, USA. A core collection of 160 accessions was developed based on 22 phenotypic traits collected among 600 accessions [15], and its genetic diversity was assessed through amplified fragment length polymorphism markers [16]. Besides, the core collection was studied to estimate biochemical conversion to ethanol [17]. Recently, part of the bermudagrass core collection [15] was included in an experiment studying nitrogen use efficiency (NUE) in bermudagrass [18]. Authors found that several traits related to NUE had large genetic variability. Nevertheless, the genetic diversity for other nutritive value (NV) traits, as well as the determination of genetic parameters, remain unknown for bermudagrass [15–18].

Nutritive value traits, especially digestibility and crude protein (CP), are main targets in forage breeding [11]. Improving NV can increase animal performance, reduce need for supplemental feed and thus cost of production, and can help mitigate some of the current environmental challenges, such as greenhouse gas emissions and eutrophication of surface waters [19–21]. Greater digestibility, for example, can lead to increase in dry matter intake and animal performance, therefore reducing methane emission per kg of animal output [22,23]. Because of high nutrient absorption capacity, bermudagrass pastures are widely used for nitrogen and phosphorus loss mitigation in waste management lands, e.g., for application of liquid and solid cattle manure, broiler litter, and other industrial water [24–27].

Phenotypic improvement requires genetic variability and stability of traits. Estimated genetic variance for dry matter digestibility in bermudagrass has been reported high (coefficient of genetic variation: 4.1–8.5%; broad sense heritability: 0.27 to 0.78), showing the potential for selection and improvement [10]. Although gains are slow, the final impact of improving NV traits can be significant. "Grazer" bermudagrass showed between 3.7 and 4.8% improvement in digestibility, which represented between 6 to 11% increase in live weight gain [28]. Increasing NV without reducing forage yield is a constant challenge for forage breeders, especially when targeting improvement for multiple traits. In 60 bermudagrass accessions from different geographic regions in China, [29] reported a phenotypic correlation of −0.37 between CP and forage yield. Tifton 85, for example, has higher digestibility and higher dry matter yield compared to Coastal, Tifton 44, 'Tifton 78', 'Jiggs', and 'Vaquero' [4,5,30–33]. However, it has lower CP compared to Jiggs [33] and greater neutral detergent fiber (NDF) concentration, which can result in low voluntary dry matter intake [34,35]. In complement, the target to increase CP, results in a decrease in NDF, whereas CP and dry matter digestibility shows a positive correlation [23].

The USDA-NPGS *Cynodon* germplasm collection has phenotypic data that have never been used for estimating variance components and genetic parameters. The estimation of genetic parameters, such as broad sense heritability (H^2), genotype by harvest interaction (GHI), and *type-A* genetic correlations, are fundamental to define breeding strategies [36]. The potential genetic diversity present in bermudagrass germplasm for traits with moderate-high H^2 and low GHI might expand the use of this germplasm by forage breeders. Hence, the objectives of this study were: (i) estimate genetic parameters for NV in the USDA-NPGS *Cynodon* collection, (ii) predict genetic values for four NV traits for the whole population across two harvests, (iii) estimate GHI for four NV traits across 11 harvests in a selected population, and (iv) select accessions exhibiting improved NV traits compared to Tifton 85.

2. Materials and Methods

2.1. Germplasm

A set of 286 bermudagrass accessions were screened, including 146 *Cynodon* clonal accessions from the USDA-NPGS forage core collection maintained in Tifton, GA; and 137 from the USDA-NPGS *Cynodon* collection maintained in Griffin, GA, and 3 commercial cultivars: Tifton 85, Coastal, and Jiggs.

Planting material obtained from Griffin and Tifton were grown in a greenhouse in Gainesville, FL, USA, and the plugs were allowed to established in 5 × 5 cm containers prior to planting.

2.2. Location Description

The experiment was conducted at the Plant Science Research and Education Unit located in Citra, Florida (29°24′16″ N and 82°10′17″ W), at 60 m elevation, from 2015 to 2016. Historical weather data was extracted online from the Florida Automated Weather Network (https://fawn.ifas.ufl.edu) and is summarized in Figure 1.

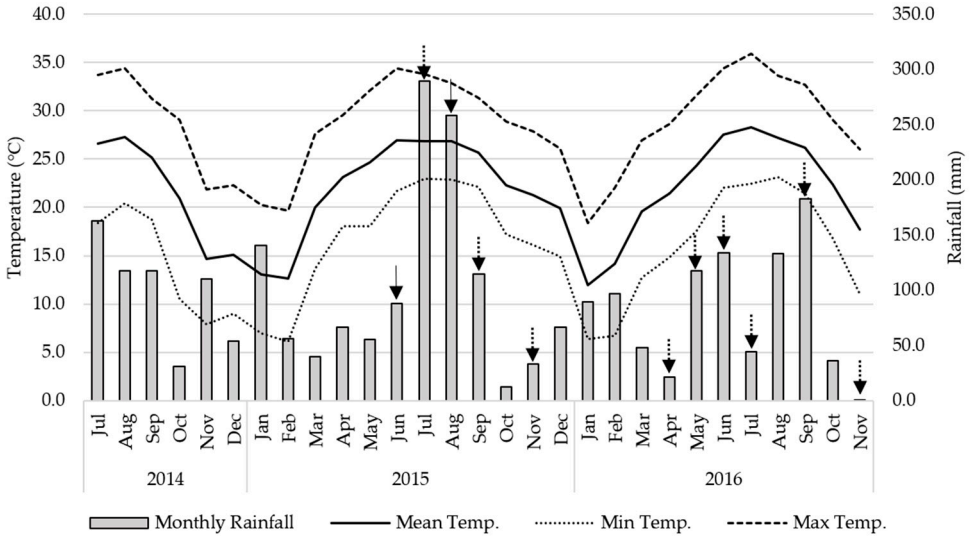

Figure 1. Maximum (Max Temp.), Minimum (Min Temp.), and Mean (Mean Temp.) monthly temperature (°C) and rainfall (mm) during the experimental period (April 2015 to November 2016). The arrows show the nutritive values sampling dates. The integer line refers to the whole population assessment, whereas the dashed line shows sampling date for the selected population.

The soil was a Chipley sand (thermic, coated Aquic Quartzipsamments) with a pH of 6.9 and characterized by high P_2O_5 content (164 kg ha^{-1}), and low K_2O (38.2 kg ha^{-1}), S (5.6 kg ha^{-1}), and Mg (44.8 kg ha^{-1}) content. The experimental plot size was 1.8 m × 3.0 m. Planting was done on 2 July 2014 using a single plug 5 × 5 cm planted in the center of the plot, and it was allowed to grow up to cover the plot. The long period between planting and the beginning of evaluations was necessary to guarantee a suitable plot establishment. The plants were fertilized with 40 g plant^{-1} with a mix of nitrogen (N), phosphorous (P_2O_5) and potassium (K_2O) 15-0-15 and micronutrients. An additional 75 kg ha^{-1} of nitrogen (N) and 45 kg ha^{-1} of K_2O were applied two months after planting. In early spring 2015, 90 kg ha^{-1} of N and K_2O were applied to promote spring regrowth. The field was re-fertilized with 90 kg ha^{-1} of N and 45 kg ha^{-1} of K_2O after each harvest, except for the last harvests of the growing seasons.

2.3. Experimental Design and Data Collection

The trial was established as a row-column design with two replicates and augmented representation of three controls, and the cultivars Tifton 85 and Jiggs were replicated thirteen times, and Coastal twelve times, in total. The plots were mowed to a stubble height of 10 cm in the beginning of each year (24 March 2015, and 3 March 2016). Plots were harvested every five weeks from April to November in both years. Biomass was collected to a 5-cm stubble height from a 1.2 × 3.0 m area in each plot. The remaining areas non-harvested were mowed to the same stubble after data collection. The fresh samples were weighed, and sub-samples (approximately 450 g) were taken, dried in a forced-air oven at 55 °C for 72 h, and reweighed to estimate forage harvested (FH). The samples were cleaned using

sieves to avoid sand contamination and ground to 1 mm using a Wiley Mill (Model 4, Thomas Scientific, Swedesboro, NJ, USA) for NV analysis.

The analyses were performed using two subsets: (i) Whole-population: all accessions were harvested twice, first on June 1st and then in 11 August 2015; and (ii) Selected-population: a selected group of 15 accessions was sampled nine extra times between 1 June 2015 to 1 November 2016. The selected group resulted from a multi-location evaluation for biomass yield and other agronomic traits. The FH predictor values only were used to elucidate genetic correlations with NV traits. The NV traits included concentration of crude protein (CP), phosphorous (P), in vitro digestible organic matter (IVDOM) and neutral detergent fiber (NDF). These traits were determined by wet chemistry at the Forage Evaluation Support Laboratory, University of Florida (FESL). Crude protein was calculated as nitrogen multiplied by 6.25. Nitrogen and P samples were digested using a modification of the aluminum block digestion procedure [37]. Sample weight was 0.25 g, catalyst used was 1.5 g of 9:1 K_2SO_4:$CuSO_4$, and digestion was conducted for at least 4 h at 375 °C using 6 mL of H_2SO_4 and 2 mL H_2O_2. Nitrogen in the digestate was determined by semiautomated colorimetry [38]. In vitro digestible organic matter was performed by a modification of the two-stage technique [39]. Neutral detergent fiber was determined using the filter bag technique according to ANKOM^{200} (ANKOM Technology, Macedon, NY, USA) procedure [40].

2.4. Statistical Analyses

A dataset for the whole-population recorded traits of the two harvests were analyzed using linear mixed models with repeated measures implemented in ASReml-R (VSNI, Hemel Hempstead, Hertfordshire, UK) [41] using the R software [42]. The following model was fitted for single-trait and multi-harvest data:

$$y = 1\mu + X_1\alpha + X_2\beta_\alpha + Z_1 g + Z_2\alpha g + Z_3 r_\beta + Z_4 c_\beta + e, \quad (1)$$

where μ is the overall population mean; 1 is a vector of ones; X_1, X_2, Z_1, Z_2, Z_3, and Z_4 are design matrices; α is the fixed harvest effect; β is the fixed effect of blocks; g is the random effects of entries, with $g \sim$ multivariate normal distribution (MVN) $(0,\sigma^2_g I)$; $g\alpha$ is the random interaction effect between entry and harvest, with $g\alpha \sim$ MVN $(0,\sigma^2_{g\alpha} I)$; r_β and c_β are the random effects of row and column nested into block, with $r_\beta \sim$ MVN $(0,\sigma^2_{r\beta} I)$ and $c_\beta \sim$ MVN $(0,\sigma^2_{c\beta} I)$; and e is the random errors, with $e \sim$ MVN $(0,\sigma^2_e I)$.

For each trait, the genotypic values for the entries were predicted and the variance components estimated: genotypic variance (s^2_g); variance of the genotype-harvest interaction (s^2_{gh}). The statistical significance of variance components were tested using the likelihood ratio test (LRT) with a Chi-square test with 1 degree of freedom [43]. Then, variance components estimates were used to calculate broad sense heritability (H^2) for each trait as

$$H^2 = \frac{s^2_g}{s^2_p} = \frac{s^2_g}{s^2_g + s^2_{gh} + s^2_e}, \quad (2)$$

and genotype by harvest correlation (r_{gh}) was calculated for each trait as

$$r_{gh} = \frac{s^2_g}{s^2_g + s^2_{gh}}, \quad (3)$$

where s^2_g is the estimated genotypic variance, s^2_p is the total phenotypic variance, and s^2_{gh} is the variance for the genotype-harvest interaction. The random error variance s^2_e for the multiharvest model was an average for both harvests (i.e., $s^2_e = (s^2_{e2} + s^2_{e5})/2$).

The genetic coefficient of variation (CV_g) was assessed for each trait using the following equation:

$$CVg = \left(\sqrt[2]{s_g^2}\frac{1}{\overline{X}}\right) \times 100, \quad (4)$$

where s_g^2 is the estimated genotypic variance, and $\overline{X}$ is the mean value of the trait [10].

Accuracy refers to the correlation between the parametric genetic value and predicted genetic value, and it considers the residual variation, the settled experimental design and the proportion between the genetic and residual variations associated with the trait under evaluation [44]. Accuracy (*Acc*) was estimated as an average based on the standard error of predicted genotypic value (*PVse*) of each genotype [44]. *PVse* is related to accuracy through the equation:

$$Acc = \sqrt[2]{\left(1 - \left((PVse^2)/s_g^2\right)\right)}, \quad (5)$$

where s_g^2 is the genotypic variance. Reliability (Rel) was obtained as average of genotypes *Acc* elevated to square. Overall mean, maximum, and minimum of predicted genotypic value were also computed. Additionally, predicted genetic values obtained with single-trait models were used to rank populations for each trait, which are given in Supplementary Table S1. A Principal Component Analyses (PCA) was performed with the *prcomp* function in R using a correlation matrix of the genotypic values obtained with the multiharvest model and predicted values of FH. These values were also used to obtain genetic correlations by Pearson method using *cor* function in R.

For the subset of selected population composed by 15 genotypes evaluated across 11 harvests, a linear model was fit for analysis of variance (ANOVA) using the package *agricolae* [45], with genotype, harvest, genotype-harvest interaction, and replicate factors considered fixed effects. A Tukey-Honest Significant Test was used to separate treatment means at $p = 0.05$ using the package *agricolae* [45], and graphs were created with the package *ggplot2* [46] in R. The coefficient of variation was calculated as standard deviation divided by mean multiplied by 100 and expressed as percentage. Additionally, a performance index was calculated by counting the number of times each genotype placed in the top of the ranking for each trait, and their mean response was statistically superior compared to any other genotype.

3. Results

3.1. Whole-Population

The whole *Cynodon* population showed significant genetic variability for all NV traits. Genetic variances for each trait were higher than zero ($p < 0.001$) based on LRT (Table 1), while GHI was only significant for IVDOM ($p < 0.001$). Genotype by harvest correlations were high for all traits (Table 1), and the H^2 ranged from low (CP and IVDOM) to moderate (P and NDF). The genetic coefficient of variation was low and exhibited a range from 2.6 to 9.6% (Table 1). Accuracy were moderate for CP, and high for other traits, whereas reliability was low for CP and moderate for the other traits.

Some traits exhibited large genotypic variability (Figure 2). For instance, CP ranged from 116 g kg^{-1} to 157 g kg^{-1} (Figure 2A), where PI 2922601, PI 292508, PI 547109, and breeding line 319 exhibited CP higher than 150 g kg^{-1}. The highest predicted value for *p* was almost double its lowest concentration. However, only four accessions, including PI 316507, Breeding line 240, 'Florakirk', and PI 364485 showed *p* higher than 3.5 g kg^{-1}, while Tifton 85 and Jiggs presented 2.9 and 3.4 g kg^{-1}, respectively, and all above the population mean (Figure 2B). Similarly, accessions exhibited wide IVDOM values from 363 to 563 g kg^{-1} (Figure 2C). 'Tifton 84' presented the highest IVDOM followed by Florakirk (555 g kg^{-1}), PI 316507 (549 g kg^{-1}), PI 255450 (548 g kg^{-1}), PI 204438 (546 g kg^{-1}), and Tifton 85 (546 g kg^{-1}). The NDF ranged from 651 g kg^{-1} to 767 g kg^{-1} (Figure 2D), where only PI 297827, PI 287156, 319, and Florakirk showed values below 660 g kg^{-1}. Despite the fact that NDF presented the lowest genotypic range and lowest genetic coefficient of variation (low genetic variability), NDF had

medium broad sense heritability and high genotype by harvest correlation. Comparing the population mean with Tifton 85, CP, P, and NDF values were similar, but Tifton 85 exhibited a much higher IVDOM compared to the population mean and most other accessions (Figure 2).

Table 1. Genetic parameters for the United States Department of Agriculture National Plant Germplasm System (USDA-NPGS) bermudagrass germplasm collection evaluated in Citra, FL. Genetic coefficient of variation (CV_g), broad sense heritability ($H^2 \pm$ SE), and genotype by harvest correlation (r_{gh}) for crude protein (CP), phosphorous (P), In Vitro Organic Matter Digestibility (IVDOM), and Nutrient Detergent Fiber (NDF) expressed at grams per kilogram. Likelihood Ratio Test of Genotype and Genotype by Harvest, Reliability Mean, and Accuracy Mean for nutritive value (NV) traits.

	Nutritive Value Traits			
	CP g.kg^{-1}	**P g kg^{-1}**	**IVDOM g kg^{-1}**	**NDF g kg^{-1}**
CV_g	5.9	9.6	8.1	2.6
$H^2 \pm$ SE	0.20 ± 0.04	0.41 ± 0.03	0.36 ± 0.04	0.40 ± 0.03
r_{gh}	0.81 ± 0.15	0.97 ± 0.07	0.81 ± 0.07	0.99 ± 0.00
Genotype (LRT)	25.9 ***	105.6 ***	72.4 ***	113.5 ***
Genotype × Harvest (LRT)	0.91 ^{ns}	0.13 ^{ns}	9.93 ***	-7.23×10^{-6} ^{ns}
Reliability Mean	0.35	0.61	0.57	0.60
Accuracy Mean	0.57	0.79	0.75	0.77

*** Significative at 0.1% by Likelihood ratio test. ^{ns}, non-significative.

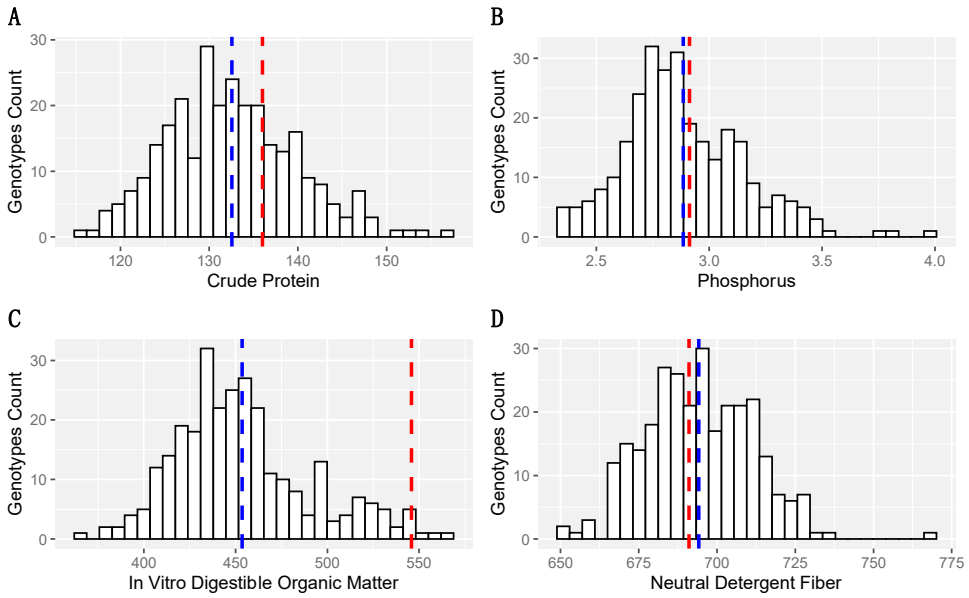

Figure 2. Histogram for four nutritive values traits using predictive values estimated for the USDA-NPGS bermudagrass germplasm collection and three checks: Tifton 85, Jiggs and Florida 44, evaluated in Citra, FL. (**A**) Crude Protein concentration, (**B**) Phosphorus concentration, (**C**) In Vitro Organic Matter Digestibility, and (**D**) Neutral Detergent Fiber, all expressed in g kg^{-1}. The dashed blue line shows the means, while the dashed red line shows the values for Tifton 85 of each NV trait.

The Pearson genetic correlations among the NV traits were all significant ($p < 0.05$), although FH was only correlated with IVDOM (Table 2). The NDF presented moderate negative correlations with CP, P, and IVDOM (Table 2). In contrast, positive correlations were observed among CP, P, and IVDOM, as well as between IVDOM and FH, in a moderate to low magnitudes.

Table 2. Pearson genetic correlations among forage harvested (FH) and nutritive value traits: CP—Crude Protein concentration, P—Phosphorus concentration, IVDOM—In Vitro Digestible Organic Matter, and NDF—Neutral Detergent Fiber; estimated for the USDA-NPGS bermudagrass germplasm collection and checks: Tifton 85, Jiggs and Florida 44, evaluated in Citra, FL. Color code: red and blue indicate negative/positive significant ($p < 0.05$) correlations, while white shows non-significative correlations.

	CP	P	IVDOM	FH
NDF	−0.68	−0.62	−0.41	−0.01
CP		0.60	0.29	−0.12
P			0.46	0.14
IVDOM				0.46

The PCA using predictive values for all accessions explained a large part of the variation found for NV traits and FH in this collection (Figure 3A). The first two PCAs accounted for 77.8% of the existing variation in the bermudagrass germplasm collection. The first principal component (PC) (eigenvalue of 2.57) explained 51.4% of variance, where the NV traits contributed more than the FH, and exhibited similar magnitudes (Figure 3A). The second PC exhibited an eigenvalue of 1.31, explained 26.41% of the variation, and the main contribution was FH (0.77), whereas other traits were less determinant, such as IVDOM (0.46), CP (−0.37), NDF (0.21), and P (−0.04). Other remaining PCs accounted for 22.2% of variation and had eigenvalues lower than 0.40.

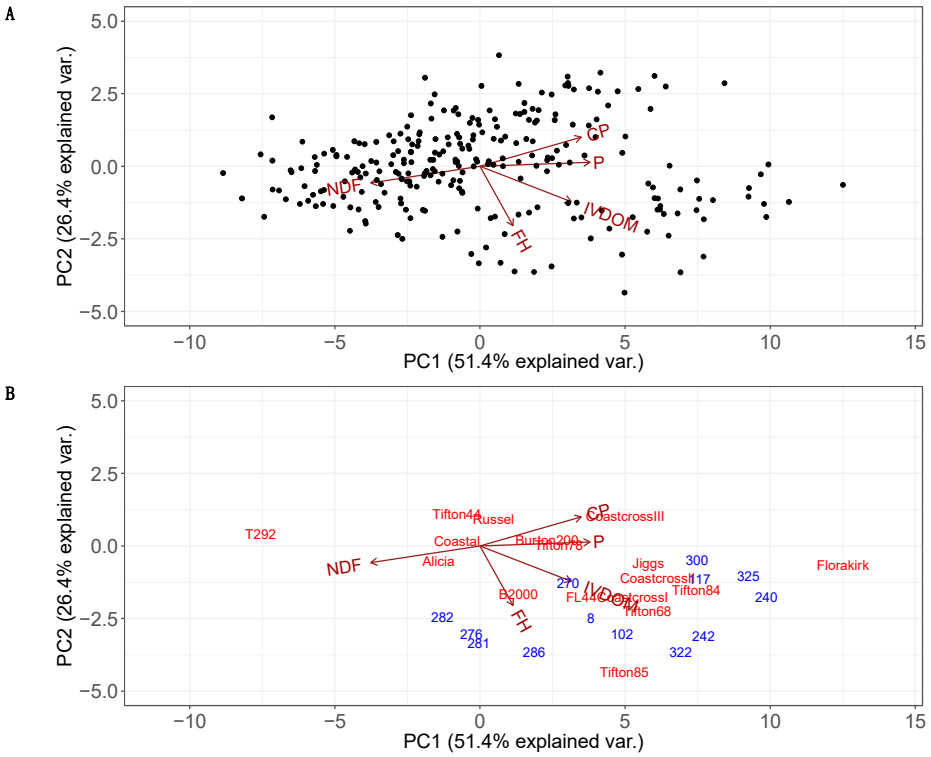

Figure 3. Principal Components Analysis (PCA) estimated for the USDA-NPGS bermudagrass germplasm collection and commercial cultivars. Black loadings represent predicted values for five traits estimated for the whole collection (**A**), and loadings colored only for 17 cultivars (red) and 15 selected accessions (blue) (**B**). The plants were grown at Citra, FL, and five traits were determined using wet chemistry: crude protein concentration—CP, phosphorus concentration—P, in vitro digestible organic matter—IVDOM, neutral detergent fiber—NDF, and forage harvested—FH. The selected accessions were identified by field ID instead of plant introduction numbers to improve visibility in the graph.

Commercial cultivars (red) and the selected accessions (blue) also exhibited broad variation (Figure 3B). Two cultivars, T292 and Florakirk, exhibited contrasting performance for NV and FH

(Figure 3B). Besides, most commercial cultivars showed lower NDF and higher values for CP, P and IVDOM in general. Tifton 85 exhibited the highest yield and IVDOM across cultivars in those two harvests. Comparing accessions and cultivars, some selected accessions exhibited higher combining of FH, IVDOM, CP, and P than commercial varieties, except by Tifton 85 and Florakirk. Tifton 85 presented high FH, while Florakirk presented high P and IVDOM. The accessions PI 316510 (322) and PI 255450 (242) grouped near Tifton 85, exhibited similar FH but higher IVDOM. In addition, some accessions exhibited high CP and P, such as PI 316507 (323), Breeding line 240 (240), PI 316536 (325), and PI 364484 (117).

3.2. Selected-Population

Significant genotype and harvest main effects ($p < 0.001$) were observed for all traits, but two-way interactions were non-significant for all traits (Table 3). For CP and IVDOM, the mean square values for harvest were considerably larger than for genotype, suggesting a higher environmental influence for these traits without influencing the genotypic response. However, the mean square value of harvest for P was lower than for genotype, which means a greater influence of genotype on P. The CV was higher for CP and P than for IVDOM and NDF but lower than 30%, which is the maximum threshold for field experiments [47]. Additionally, NDF exhibited CV lower than 5%, which was similar to the CV_g observed in the whole population.

Table 3. Analysis of variance showing sources of variation, degrees of freedom (df), and mean square values for each nutritive value trait.

Source of Variation	df	Mean Square Values			
		CP g kg^{-1}	P g kg^{-1}	IVDOM g kg^{-1}	NDF g kg^{-1}
Replicate	1	0.15 ^{ns}	0.00019 ^{ns}	1.2 ^{ns}	44.47 ***
Genotype	14	16.66 ***	0.05361 ***	284.2 ***	98.38 ***
Harvest	10	91.29 ***	0.02211 ***	511.4 ***	85.93 ***
Harvest by Genotype	140	2.46 ^{ns}	0.00178 ^{ns}	14.0 ^{ns}	2.51 ^{ns}
Error	170	3.64	0.0017	14.3	3.78

*** $p < 0.001$; ^{ns}, non-significant differences.

Tifton 85 and Florida 44 were not different for CP between themselves or from other accessions (Table 4). However, PI 308193 had higher CP compared to PI 255456, PI 255450, PI 290813, PI 290664, and PI 295114 (Table 4). PI 316510 had similar CP than PI 308193, and it was higher than PI 290813, PI 290664, and PI 295114. For phosphorus content, Tifton 85 and Florida 44 performed similarly, and Tifton 85 was not statistically different from the PIs with lowest P. Additionally, Breeding line 240 and PI 255450 presented the highest P.

The accessions PI 316510, Breeding line 240, Breeding line 8, Tifton 85, and PI 316536 had similar IVDOM. Finally, breeding line 240 and PI 364484 exhibited the lowest NDF but were not significantly different from PI 316510, PI 316536, PI 255456, and Florida 44. All these PIs presented lower NDF than Tifton 85. Based on a performance index (Table 4), breeding line 240 was placed at the top of the ranking for the four NV traits, while PI 316510, PI 316536, and Breeding line 8 performed well for CP, IVDOM, and NDF. Breeding line 240 presented higher P than PI 316510, PI 316536, and Breeding line 8, as well as lower NDF than Breeding line 8. These accessions had lower NDF and higher P than Tifton 85. The PI 255450 showed high P concentration and exhibited high IVDOM similar to PI 316510. Finally, NDF presented the largest difference between the selected accessions and Tifton 85 (Table 4).

Table 4. Average nutritive value for selected bermudagrass plant introductions (PI), breeding lines, and cultivars across eleven harvests evaluated in Citra, FL.

Entries	CP g kg^{-1}		P g kg^{-1}		IVDOM g kg^{-1}		NDF g kg^{-1}		Performance Index *
PI 308193 (300)	145	a *	3.2	bc	467	fg	678	cd	1
PI 316510 (322)	139	ab	3.2	bc	557	a	662	de	3
PI 316536 (325)	139	abc	3.1	bcd	529	abcd	670	de	3
PI 364484 (117)	134	abcd	3.4	b	503	cdef	656	e	1
Breeding line 240 (240)	133	abcd	4.0	a	551	ab	654	e	3
Breeding line 8 (8)	133	abcd	2.5	ef	543	abc	691	bc	1
PI 292143 (102)	127	abcd	3.2	bc	516	bcde	679	cd	0
PI 294467 (276)	125	abcd	2.6	ef	488	efg	708	ab	0
PI 255456 (270)	124	bcd	3.4	b	499	defg	669	de	1
PI 255450 (242)	122	bcd	3.9	a	564	a	680	cd	2
PI 290813 (286)	119	cd	2.4	f	483	efg	708	ab	0
PI 290664 (281)	115	d	2.5	ef	479	efg	716	a	0
PI 295114 (282)	115	d	2.4	f	463	fg	719	a	0
Florida 44	127	abcd	2.9	cde	461	g	667	de	1
Tifton 85	133	abcd	2.8	def	541	abc	693	bc	1
C.V. (%)	19		22		12		5		

Mean values with same letter do not differ statistically ($p \leq 0.05$) by Tukey Test. * Performance index: number of times a genotype appear in the top statistical group for each trait.

The NV traits varied greatly across harvests (Table 5). The NV fluctuated within the year, with higher CP and lower NDF in the April and September harvests. Higher IVDOM values were measured in the spring and beginning of summer for both years, coinciding with high volumes of rainfall and mild temperature (Figure 1). Lower values for CP, IVDOM, NDF, and P were observed in November, with scarce rainfall and low temperature inducing dormancy. The NV measured on the same month on both years were similar, and for some traits, there were no significant differences (Table 5). Although the fluctuations in temperature observed during both years were similar, the rainfall regime was different (Figure 1). In 2015, July and August had 250 mm per month, which was above average, whereas the rainfall the same period in 2016 was less than 133 mm per month. The rainfall in June was 290 mm in 2015, whereas it was 43 mm in 2016.

Table 5. Average nutritive value by harvest (11) for the selected population (15 entries).

Harvest	CP g kg^{-1}		P g kg^{-1}		IVDOM g kg^{-1}		NDF g kg^{-1}	
2015 June 1	124	d *	2.9	cd	556	a	694	bc
2015 July 7	117	d	3.0	abc	536	ab	692	bcd
2015 August 11	85	e	2.4	e	535	ab	699	b
2015 September 22	151	a	3.3	ab	518	bc	675	ef
2015 November 3	128	bcd	3.0	abc	448	e	686	bcde
2016 April 14	150	a	3.0	bcd	558	a	652	g
2016 May 19	120	d	3.1	abc	549	a	719	a
2016 June 23	129	bcd	3.3	a	509	bcd	678	def
2016 July 28	140	ab	3.2	abc	488	cd	681	cdef
2016 September 13	138	abc	3.2	abc	483	d	670	f
2016 November 1	125	cd	2.6	de	436	e	677	def
C.V. (%)	19		22.3		12.4		4.6	

* Mean values with same letter do not differ statistically ($p \leq 0.05$) by Tukey Test.

4. Discussion

The genetic parameters indicated that genetic gains can be expected in all NV traits in this bermudagrass germplasm, as most traits presented moderate H^2. Broad sense heritability explains the magnitude of phenotypic variance due to the genetic variance [36]. Indeed, a higher genetic variance than GHI was observed for all traits, and the GHI was only significant for IVDOM. GHI effect was

considerably lower than the main genotypic effect, similar to previous reports in maize (*Zea mays* L.) and timothy grass (*Phleum pratense* L.) [48,49], if we treat harvest as an environment. Thus, high stability of genotypes for these traits across harvests would be expected as supported by the high estimates of r_{GH}. The use of r_{GH}, although it does not elucidate the significance of variance sources, indicates the stability of genotypes among harvests in a given location, meaning that traits with high stability can be subjected to selection with fewer harvests. Given the high r_{GH} for all traits, using estimates from fewer harvests can be a reliable NV measure to screen large nurseries in forage breeding programs. However, considering a selection for multiple locations, other environmental variables, such as soil type, crop management, and climate variability, might diverge and cause genotype by environment interaction (GEI). Therefore, we encourage other studies to elucidate GEI for NV traits. Despite that, using only two harvests for the whole population, it was possible to achieve prediction accuracies higher than 0.70 for IVDOM, NDF, and P, considered appropriate for forage breeding experiments [50]. In general, an increase in the number of harvests leads to an increase in precision; however, it adds costs and labor when dealing with large populations [51].

Genetic parameters, such as H^2, r_{GH}, and CV_g, are variable among species, germplasm, traits, and experiments [52]. Medium to high broad-sense heritability for IVDOM have been reported for other forage grasses, 0.56 to 0.93 [53–55]. Previous efforts with bermudagrass showed broad-sense heritability ranging between 0.27 and 0.78 across years and experiments [11], denoting that H^2 is inherent to the experimental conditions, as well as in where moderate to high estimates can be achieved for those species. In our study, H^2 estimates were within the range of previous reports. The magnitude of H^2 for P in the bermudagrass collection was similar to the broad-sense heritability of 0.46 reported for reed canarygrass (*Phalaris arundinacea* L.) [56] but lower than the 0.72 estimate reported in tall fescue (*Schedonorus arundinacus* (Schreb.) Dumort.) [57].

In turn, NDF had lower H^2 compared to previous studies, and lower variation (CV_g) in the population. Broad-sense heritability in NDF for populations of tall fescue [54] and koronivia grass (*Urhochloa humidicola* (Rendle), Morrone and Zuloaga [58]) were much greater (0.85 and 0.58, respectively) compared to the bermudagrass germplasm. Other reports also showed high narrow-sense heritability for NDF in tall fescue (0.6; Reference [59]) and signal grass (*Urochloa decumbens* (Stapf) R. Webster) (0.74; Reference [51]). Moreover, r_{GH} across multiple harvests was also lower for the referred studies (0.36 in koronivia grass [58]; 0.82 in signal grass [51]). Thus, the efforts to genetically improve NDF from this germplasm would be similar to the above-mentioned species but fewer evaluations are needed since it has higher stability.

Crude protein exhibited lower H^2 than the estimates for the other NV traits, but similar H^2 estimates were found in other species. Low broad-sense heritability was reported for CP in meadow fescue (0.21) [53], as well as low narrow-sense heritability (0.07) in koronivia grass [58], 0.14 in congo grass [60], and 0.18 in tall fescue [59]. The estimates of H^2, also, can be influenced by environment. In tall fescue, narrow heritability ranged from 0.18 to 0.54, with the winter harvest exhibiting a higher estimate than that for the fall harvest [59]. Thus, the choice of the harvest to characterize CP is important because managing data collection can minimize undesirable effects attributed to the large environmental variance compared to measured genetic variance in this study. Although CP had low H^2 that will potentially result in lower genetic gains for this trait, any gain in protein content in grasses can result in significant reductions of the use of external protein sources. The high stability of this trait over the two annual harvests (high r_{GH}), and non-significant GHI indicated that most of the variation in CP in this population was indeed due to genetics.

The genetic coefficient of variation complements the broad sense heritability estimates, and it allows the comparison of genetic variability among populations and traits [10]. The CV_g for IVDOM was 8.1%, which is higher than values reported for meadow fescue (4%) [53], congo grass (2.2%) [61] and *Urochloa* spp. (5.8%) [55]. Digestibility has been a major target in previous bermudagrass breeding efforts (e.g., Coastcross I; Reference [11]. In an effort to improve digestibility in bermudagrass, approximately 500 accessions showed great variability for in vitro dry matter digestibility (IVDMD) and

CV_g ranged from 6.1 to 28.9%, and the two-year average for IVDMD ranged from 400 to 690 g kg^{-1} [10]. This large genetic variation can be exploited in breeding programs. In our study, the PI 255450 (242), which is a parental line for Kenya 61 [11], exhibited the highest IVDOM. Although it could be used in crosses aiming to combine higher digestibility with other traits, the ploidy level of this accession can be a limitation because the progeny from Coastal and Kenya 61 were all male sterile and not able to produce seeds [11]. Other Pis, such as Tifton 84, Florakirk, and PI 316507, also showed high IVDOM.

Phosphorous had the largest CV_g among the NV traits in this population. Breeding line 240 and PI 316510 (322) had an average 30% more P concentration than Tifton 85, as well as desired levels of CP, IVDOM, and NDF. This genetic variability can be explored to generate information about P uptake and its relationship with forage yield, as previous research reported for N [18]. Accessions with high uptake P and N that combine reasonable agronomic performance can be used as phytoremediation agents for dairy farms [27,62]. On the other hand, genetic variability for NDF was the lowest among evaluated traits, concurrent with other reports from the literature, like 1.4 and 2.9% in tall fescue [59], 3.8 to 4.2% in leaves of *Brachiaria* spp. [55] 4.32, and 4.43% in leaves and stems of *Arachis* spp. [59]. These aspects confirm that difficulty in improving NDF by conventional breeding in forage species.

Entries with high NV traits were already included in the bermudagrass core collection except for four accessions (PI 292508, PI 547109, PI 297827, and PI 287156-01), as selection was based on FH across several environments [15]. Breeding line 240, PI 316510 (322) and PI 3166536 (325) presented lower NDF and greater P concentration than Tifton 85, and similar IVDOM and CP. Breeding for improved CP has received little attention in the past as nitrogen fertilizer has been an affordable and effective way to increase increasing CP concentration, IVDOM, and productivity in grasses [17,18,23]. Few studies looked at nitrogen use efficiency in bermudagrass [18], which found high variability for the trait, and a negative correlation to CP concentration especially at low N fertilization rates.

In general for bermudagrass, CP has negative correlation with biomass and NDF (−0.32 and −0.36, Reference [29]), but positive correlation with dry matter digestibility (0.34; [11]). Similar results were observed on a review of over one hundred forage species, where digestibility had a positive correlation with CP (0.62) and negative with NDF (−0.68) [63]. Our results showed similar trends, although FH was only significantly correlated to IVDOM. The moderate to low genetic correlations found in the current germplasm suggest genes related to the expression of these traits have higher independence or belong in different metabolic routes. This can be due to the genetic diversity present in this germplasm, particularly related to having different species and ploidy levels in the core collection [15,16]. Thus, breeding multiple nutritive value traits in *Cynodon* ssp. should be achievable, and there is still potential for NV to be improved with forage yield.

Selection on a single trait provides higher genetic progress but can be detrimental if some unwanted correlation exists with the trait under improvement. On the other hand, genetic correlations can be used to identify potential indirect selections for simultaneous breeding [36]. Although there was no clear cluster of genotypes in the PCA, it was possible to identify genotypes that presented superior NV traits and FH. For instance, except for Tifton 85 and Florakirk, other accessions in the selected population presented higher P, IVDOM, and FH than most commercial cultivars. In general, commercial varieties and the selected population showed variability and different patterns of NV performance. Accessions such the breeding line 240, PI 255450, and PI 316510 exhibited high FH and NV. Thus, they can be selected as parental lines in future crosses and public cultivar releases.

5. Conclusions

The results presented in this study complement previous findings and provide useful information for the entire forage bermudagrass collection, aiming at developing cultivars with improved NV. The high significance of the genotypic factor evaluated during eleven harvests showed differences among the accessions in the selected-population for all NV traits. These accessions have a good combination of NV and biomass production, and some of them had improved NV compared to Tifton 85. The lack of significant two-way interactions between genotype and harvest for NV traits

confirmed the high stability of genotypes across harvests. Thus, selection for NV can be assessed with fewer harvests, resulting in savings for time, labor, and resources. Genetic parameters revealed that P has higher potential to be explored as breeding target, along with CP, while narrow variance for NDF, and the availability of varieties with high IVDOM, would require more effort than conventional breeding for improving those traits in bermudagrass. Breeding line 240, PI 316510 (322), and PI 3166536 (325) presented superior nutritive value than Tifton 85 and will be considered for public cultivar releases in the United States.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/11/1729/s1, Table S1: Predicted genetic values estimated of nutritive value traits: crude protein (CP), phosphorous (P), in vitro digestible organic matter (IVDOM) and nutrient detergent fiber (NDF) in $g.kg^{-1}$, for the USDA-NPGS bermudagrass germplasm collection evaluated in Citra, FL.

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