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Genetic Diversity and Population Structure of *Brachiaria* (syn. *Urochloa*) Ecotypes from Uganda

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Abstract: *Brachiaria* (syn. *Urochloa*) grass is an important tropical forage of African origin that supports millions of livestock and wildlife in the tropics. Overgrazing, conversion of grasslands for crop production and non-agricultural uses, and the introduction of improved forages have threatened the natural diversity of *Brachiaria* grass in Uganda. This study established a national collection of *Brachiaria* ecotypes in Uganda and analyzed them for genetic diversity and population structure using 24 simple sequence repeats (SSR) markers. These markers had a high discriminating ability with an average polymorphism information content (PIC) of 0.89 and detected 584 alleles in 99 ecotypes. Analysis of molecular variance revealed a high within populations variance (98%) indicating a high gene exchange or low genetic differentiation (PhiPT = 00.016) among the ecotype populations. The Bayesian model based clustering algorithm showed three allelic pools in Ugandan ecotypes. The principal component analysis (PCA) of ecotypes, and Neighbor-joining (NJ) tree of ecotypes and six commercial cultivars showed three main groups with variable membership coefficients. About 95% of ecotype pairs had Rogers' genetic distance above 0.75, suggesting most of them were distantly related. This study confirms the high value of these ecotypes in *Brachiaria* grass conservation and improvement programs in Uganda and elsewhere.

Keywords: cluster analysis; polymorphic information content; genetic variation; forage grass; polyploidy; phylogenetic

1. Introduction

The genus *Brachiaria* (Trin.) Griseb. (syn. *Urochloa*) belongs to the tribe Paniceae in the subfamily Panicoideae of the family Poaceae [1]. It consists of about 100 species distributed throughout the tropics especially in Africa [2]. Seven perennial species of African origins—*Brachiaria arrecta* (Hack. ex. Th. Dur and Schinz) Stent, *Brachiaria brizantha* (A. Rich.) Stapf., *Brachiaria decumbens* Stapf, *Brachiaria dictyoneura* (Fig. and De Not.) Stapf, *Brachiaria humidicola* (Rendle) Schweick, *Brachiaria mutica* (Forssk.) Stapf and *Brachiaria ruziziensis* Germain and Evrard—have been used as fodder plants [3]. All *Brachiaria* species with known forage values occur naturally in eastern Africa which represents the center of diversity of the genus [3]. These agriculturally important *Brachiaria* species were introduced to other parts of the

world in multiple occasions—e.g., *B. decumbens* to Australia from Ugandan Department of Agriculture in 1930 [4,5], *B. mutica* to the Americas during the early colonial period [6] whereas, *B. decumbens*, *B. brizantha*, *B. ruziziensis*, and *B. humidicola* to the Americas between the 1950s and 1970s [7].

Brachiaria is probably the most widely distributed sown pasture in the tropics [8] with acreage of about 99 million hectares in Brazil alone [9]. *Brachiaria* is adaptable to a wide range of habitats from swamps and light forest shades to semi-deserts. However, most species are typically found in savannas [2]. *Brachiaria* grass is appreciated for adaptation to drought and low fertility soils, soil stabilization, carbon sequestration, high efficiency in nitrogen use, and lessening greenhouse gas emission [10–14]. *Brachiaria* is an ideal forage for improving livestock productivity both for cut-and-carry and grazing systems and yields between 5–36 t/ha [15]. The role of *Brachiaria* grass in the transformation of livestock agriculture has been well appreciated across the tropics, creating a high demand for improved cultivars adapted to different agroecological zones. Therefore, there is a need to develop *Brachiaria* cultivars that have superior agronomic traits such as adaptation to different agro-climatic zones, high biomass production, high nutritive values, and resistance to pests and diseases [16,17].

Evaluation of *Brachiaria* species (*B. brizantha*, *B. decumbens*, *B. ruziziensis*, and *B. mutica*) for pasture production in Africa started in the 1950s focusing on dry matter yield, nutritive value, agronomic qualities, response to inputs, compatibility with legumes and other crops, and livestock productivity [18]. Despite a broad adaptation, high nutritive value, and positive gains in livestock productivity, the use of *Brachiaria* grass for pasture production was limited because *Brachiaria* was less suitable to the main livestock production systems of Africa [18]. The recognition of the forage potential of *B. decumbens* in Australia in the 1960s stimulated the interest on *Brachiaria* grass across the tropics, specifically in South America [19–21]. The research at International Centre for Tropical Agriculture (CIAT) and the Brazilian Agricultural Research Corporation (Embrapa) on naturally occurring germplasms from Africa have developed several improved *Brachiaria* cultivars and a few interspecific hybrid cultivars for South America. Over the past few years, some of these cultivars have been evaluated for pasture production in Africa focusing on biomass production, nutritive values, livestock productivity, and adaptation to biotic and abiotic stresses [12,14,22]. Results of evaluations have shown the great potential of some *Brachiaria* cultivars in Africa to alleviate livestock feed shortage, increase the availability of quality feeds and improve livestock productivity and income of livestock farmers in Africa [12,22,23]. The underlying high agro-ecological diversity in Africa needs *Brachiaria* cultivars that are suitable for different production environments.

Uganda is an East African country that spans the Northern Hemisphere and the Southern Hemisphere. It accounts for 0.18% of the global terrestrial and freshwater surface and harbors 1.1% of the globally recognized plant species [24] substantiating a high plant species diversity in Uganda. Similarly, Uganda has diverse environmental conditions divided into eleven agroecological zones [25]. In Uganda, *Brachiaria* grows naturally on communal grazing areas, ranches, government farms, along the roadside and forests. Due to the high palatability of *Brachiaria* grass compared to most other tropical forages, the native populations are often overgrazed and are in continuous threat of extinction. Due to rapid growth in population, the natural pastures are being converted rapidly to croplands and other non-agricultural uses which present a serious risk of extinction of plant genetic resources including *Brachiaria* grass. Except CPI 1694, the accession from which the first *Brachiaria* cultivar Basilisk was derived, no *Brachiaria* germplasm from Uganda is available in the gene bank at the Consultative Group for International Agricultural Research (CGIAR) and other institutions [3]. Therefore, this study was carried out to establish a nationwide collection of *Brachiaria* grass ecotypes in Uganda, document natural genetic diversity and population structure, and determine the utility of these ecotypes for *Brachiaria* conservation and improvement programs. For this study, we used Single Sequence Repeats (SSR) markers for genetic diversity study as they are reported to be highly informative due to their multi-allelic nature, co-dominant inheritance, and wider genomic distribution [26].

These markers are proven to be the most suitable for the genetic diversity studies in *Brachiaria* grass and other forages [1,18,27–31]. This study has set up a national collection of *Brachiaria* ecotypes in Uganda, documented natural genetic diversity and population structure of ecotypes, and recognized the merits of these ecotypes in *Brachiaria* grass conservation and improvement programs.

2. Materials and Methods

2.1. Source of Plant Materials

A total of 99 *Brachiaria* grass ecotypes were collected from nine districts (Arua, Fort Portal, Hoima, Kabale, Lira, Masindi, Mbarara, Njeru, and Wakiso) representing five regions (northern, western, southwestern, central and south dryland) (Figure 1) and eight different agroecological zones in Uganda. For each ecotype, 5–10 rooted tillers were collected from a single plant, transported to National Livestock Resources Research Institute (NaLIRRI), Tororo, Uganda, and maintained as a single plant in vegetative field gene bank under the guardianship of NaLIRRI.
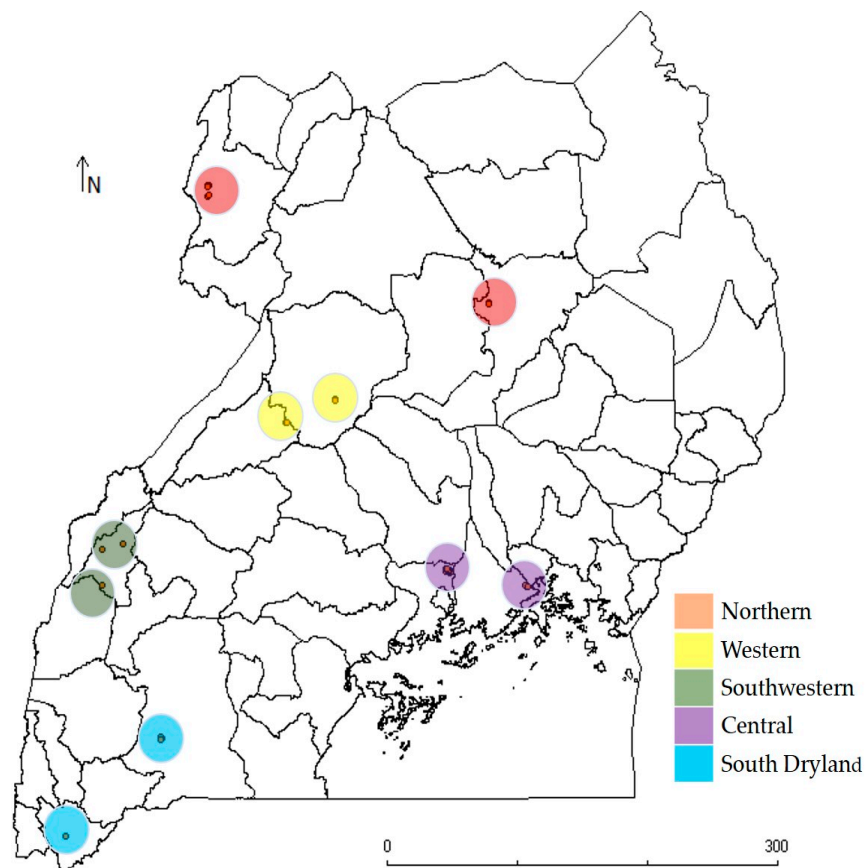

Figure 1. Map of Uganda showing *Brachiaria* ecotype collection sites in five regions (regions are coded in different colors). The map was generated using DIVA-GIS software.

The origins and details of ecotype collection sites are presented in Table S1. Two-week old leaves from 2-month old regrowth were collected separately for each ecotype for total genomic DNA extraction. Leaves were put into Ziploc bags and transported in ice chests to the Biosciences eastern and central Africa—International Livestock Research Institute (BecA-IRLI) Hub laboratory in Nairobi, Kenya. Young leaf samples of six commercial cultivars: *B. brizantha* cv. MG4, *B. brizantha* cv. Piatã, *B. decumbens* cv. Basilisk, *B. humidicola* cv. Humidicola, *B. humidicola* cv. Llanero, and *Brachiaria* hybrid Mulato-II were also included in the study. All leaf samples were freeze-dried and stored at −80 °C prior to DNA extraction.

2.2. DNA Extraction

Total genomic DNA was extracted from freeze-dried and ground leaf samples of 99 ecotypes and six commercial cultivars using the Quick-DNA Plant/Seed Miniprep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. DNA concentration and purity were determined using a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Samples were normalized to a concentration of 20 ng/μL, then run on a 1% agarose-0.5xTBE gel stained with 0.25× GelRed at 100 volts for 45 min and visualized under UV light (UVP Bio-Imaging Systems, Upland, CA, USA) to assess the integrity of DNA.

2.3. PCR Amplification and Capillary Electrophoresis

A total of 24 fluorescent-labeled SSR markers were used for genotyping (Table 1). These markers were initially developed for *B. ruziziensis*, had high polymorphism information content (PIC) values, and confirmed for transferability to other *Brachiaria* species [21]. The forward primers were labeled with the fluorescent dyes- 6-FAM, PET, NED, and VIC. Markers used in this study consisted of dinucleotide and trinucleotide repeat motifs. Gradient PCR was carried out for each primer sets and the annealing temperature that gave clear bands was identified for each primer set. Multiplex panels were designed based on annealing temperature and dye label. Each of the eight selected panels consisted of 1–4 sets of primers.

PCR reaction was performed on a total volume of 10 μL using AccuPower® PCR PreMix, negative dye (Bioneer, Daejeon, Korea), 40 ng template DNA, 0.09 μM each of forward and reverse primer, additional 0.5 μM $MgCl_2$, and 7.2 μL triple distilled water. The PCR reaction was performed in a GeneAmp PCR system 9700 thermocycler (Applied Biosystem, Foster City, CA, USA) using the following program: initial denaturation at 95 °C for 3 min, followed by 30 cycles of 30 s at 94 °C, 1 min at optimized annealing temperature for each primer pair, and 2 min at 72 °C for extension. This was followed by 20 min final extension at 72 °C and hold at 15 °C. The PCR products were run on a 2% agarose gel in 0.5x TBE buffer stained with 0.25x GelRed at 6.7 V/cm for 45 min and visualized under UV light. The size of the band was estimated using 1 kb plus ladder.

A cocktail (LH) of 15 μL GeneScan™ -500 LIZ® Size Standard (Applied Biosystems, Foster City, CA, USA) and 1000 μL Hi-Di™ Formamide was prepared for capillary electrophoresis. Multiplexed PCR product (1.5 μL) was mixed with 9 μL of LH, denatured at 95 °C for 3 min, and snapped-chilled for 5 min. The products were submitted to BecA-ILRI Hub's Sequencing, Genotyping, and Oligonucleotide (SEGOLIP) unit for capillary electrophoresis.

Table 1. Characteristic of 24 Single Sequence Repeats (SSR) markers and marker summary statistics.

Marker	Forward Primer Sequence (5′ to 3′)	Reverse Primer Sequence (5′ to 3′)	Annealing Temp. (°C)	Expected Product Size	Repeat Motif	Summary Statistics	
						No. of Allele	PIC
Br0012	ACTCAAACAATCTCCAACACG	CCCACAAATGGTGAATGTAAC	59	144–196	$(CA)_6$	14	0.91
Br0028	CATGGACAAGGAGAAGATTGA	TGGGAGTTAAACATTAGTGTTTT	58	111–197	$(TA)_8$	38	0.96
Br0029	TTTGTGCCAAAGTCCAAATAG	TATTCCAGCTTCTTCTGCCTA	59	132–178	$(CT)_{14}$	14	0.84
Br0031	CCCCCATTTAACACCATAGTT	GCTCAAAATGCAATGTACGTG	59	139–179	$(AC)_7$	21	0.93
Br0067	TTAGATTCCTCAGGACATTGG	TCCTATATGCCGTCGTACTCA	59	130–171	$(AT)_{11}$	25	0.91
Br0076	CCTAGAATGCGGAAGTAGTGA	TTACGTGTTCCTCGACTCAAC	59	120–262	$(CA)_7$	14	0.88
Br0087	TTCCCCCACTACTCATCTCA	AACAGCACACCGTAGCAACT	59	229–261	$(AT)_8$	36	0.88
Br0092	TTGATCAGTGGGAGGTAGGA	TGAAACTTGTCCCTTTTTCG	58	200–295	$(AT)_6$	14	0.76
Br0100	CCATCTGCAATTATTCAGGAAA	GTTCTTGGTGCTTGACCATT	58	229–286	$(AT)_{11}$	23	0.95
Br0115	AATTCATGATCGGAGCACAT	TGAACAATGGCTTTGAATGA	59	231–315	$(GA)_8$	28	0.93
Br0117	AGCTAAGGGGCTACTGTTGG	CGCGATCTCCAAAATGTAAT	58	233–345	$(TA)_5$	27	0.83
Br0118	AGGAGGTCCAAATCACCAAT	CGTCAGCAAATTCGTACCAC	59	237–321	$(TC)_{11}$	21	0.60
Br0122	CATTGCTCCTCTCGCACTAT	CTGCAGTTAGCAGGTTGGTT	58	223–279	$(CT)_{11}$	18	0.88
Br0130	TCCTTTCATGAACCCCTGTA	CATCGCACGCTTATATGACA	58	199–299	$(AG)_{14}$	26	0.95
Br0149	GCCAAGACCGCTGTTAGAGAA	CTAACATGGACACCGCTCTT	58	231–299	$(AT)_9$	26	0.91
Br0152	ATGCTGCACTTACTGGTTCA	GGCTATCAATTCGAAGACCA	58	233–301	$(AT)_7$	28	0.93
Br0156	GCCATGATGTTTCATTGGTT	TTTTGCACCTTTCATTGCTT	58	231–286	$(AT)_6$	28	0.95
Br0203	CGCTTGAGAAGCTAGCAAGT	TAGCCTTTTGCATGGGTTAG	58	208–310	$(GA)_9$	23	0.88
Br0212	ACTCATTTTCACACGCACAA	CGAAGAATTGCAGCAGAAGT	59	248–330	$(AAT)_{10}$	21	0.89
Br0213	TGAAGCCCTTTCTAAATGATG	GAACTAGGAAGCCATGGACA	58	212–337	$(AT)_9$	25	0.83
Br0214	TCTGGTGTCTCTTTGCTCCT	TCCATGGTACCTGAATGACA	59	241–358	$(CA)_5$	24	0.96
Br0235	CACACTCACACACGGAGAGA	CATCCAGAGCCTGATGAAGT	59	239–330	$(TC)_9$	33	0.87
Br3002	GCTGGAATCAGAATCGATGA	GAACTGCAGTGGCTGATCTT	59	143–187	$(AAT)_7$	17	0.92
Br3009	AGACTCTGTGCGGGAAATTA	ACTTCGCTTGTCCTACTTGG	58	116–199	$(AT)_8$	40	0.94
Mean						24.3	0.89

2.4. Allelic Scoring

Allele calling and sizing were performed manually using GeneMapper Software v4.1 (Applied Biosystems, Foster City, CA, USA). The SSR fragments were analyzed following a dominant scoring scheme as the information on ploidy levels for Ugandan ecotypes was not available [29–31]. Well defined clear peaks were binned according to expected product size and data was exported to Microsoft Excel for analysis. The allele calls were converted to binary data (0 = absence and 1 = presence of alleles) using ALS-Binary Software [32] for subsequent analyses. Both allelic and binary data were used in the genetic diversity analysis. The SSR genotyping data for the commercial cultivars was used in the construction of neighbor-joining tree only.

2.5. Population Genetic Analyses

The model-based clustering approach implemented in the software package STRUCTURE version 2.3.4 was used to analyze the population structure [33]. To estimate the posterior probabilities (qK), a 100,000 burn-in period was used, followed by 100,000 iterations using a model allowing for admixture and correlated allele frequencies with no prior location or population information. At least 10 independent runs of STRUCTURE were performed by setting K from 1–10 with 15 replicates for each K. The Delta K was calculated for each value of K using the web-based Structure Harvester [34,35]. A line was assigned to a given cluster when the proportion of its genome in the cluster (qK) was higher than a threshold value of 50%.

Matrices of Roger's genetic distance [36], gene diversity, PIC value, and allele frequency for each locus were calculated between each pair of lines using PowerMarker v3.2.5 [37]. The Pearson correlation coefficient matrix was computed to examine what type of linear relationships of rainfall, altitude, sample size, and the allele frequencies of regional ecotype populations had using XLSTAT software [38]. The genetic distance matrices for ecotypes and six commercial *Brachiaria* cultivars were used for constructing Neighbor-joining trees using PowerMarker and the resulting trees were visualized using MEGA version 5.0 [39]. Analysis of Molecular Variance (AMOVA) [40,41] was used to partition the variation among and within group (population) components in GenAlEx version 6.5 [42] which enabled the estimation of standardized PhiPT and the allelic patterns across different populations [43]. Significance levels for variance component estimates were computed using 9999 permutations. Principal component analysis (PCA) was performed to visualize similarities and variations among *Brachiaria* ecotypes from Uganda in DARwin software version 6.0.15 [44].

3. Results

3.1. Microsatellite Diversity and Analysis of Molecular Variance

Twenty-four SSR markers were used for genotyping 99 Ugandan *Brachiaria* grass ecotypes (Table 1) and six commercial cultivars. These markers detected 584 alleles of different sizes ranging from 111 bp (Br0028) to 358 bp (Br0214) in 99 Ugandan ecotypes (Table 1). The PIC values for these markers ranged between 0.60 (Br0118) and 0.96 (Br0028) with 0.89 average. The analysis of molecular variance (AMOVA) revealed a high contribution of within the population differences (98%) to the total genetic variations, whereas the rest (2%) was contributed by populations' differences. The genetic differentiation among the ecotype populations (PhiPT) was low (0.016) (Table 2).

Table 2. Analysis of molecular variance (AMOVA) of Ugandan *Brachiaria* ecotypes.

Source	DF	SS	MS	Est. var.	(%)
Among populations	4	942826.981	235706.745	2905.353	2%
Within populations	94	16796439.605	178685.528	178685.528	98%
Total	98	17739266.586			
Genetic differentiation among ecotype populations (PhiPT) = 0.016; p = 0.142					

Notes: DF = degree of freedom, SS = sum of squares, MS = squares, Est. var. = estimate of variance, and (%) = percentage of total variation. p-value is based on 9999 permutations.

3.2. Allelic Diversity in the Regional Populations

The allelic diversity in five regional populations of Brachiaria ecotypes is presented in Figure 2. The populations varied for mean numbers of different alleles (Na) that ranged from 2.92 (western (WST)) to 4.63 (central (CTR)). Similarly, differences were observed among the populations for the mean number of private alleles (Np) ranging from 0.674 (south dryland (SDL)) to 1.542 (CTR), as well as for the mean number of effective alleles (Ne) which ranged from 2.39 (southwestern (SWT)) to 3.33 (northern (NTN)). We detected the highest mean genetic diversity in NTN population whereas the WST population had the least mean genetic diversity (0.74). The expected heterozygosity (He) of the populations ranged between 0.36 (WST) and 0.56 (NTN).
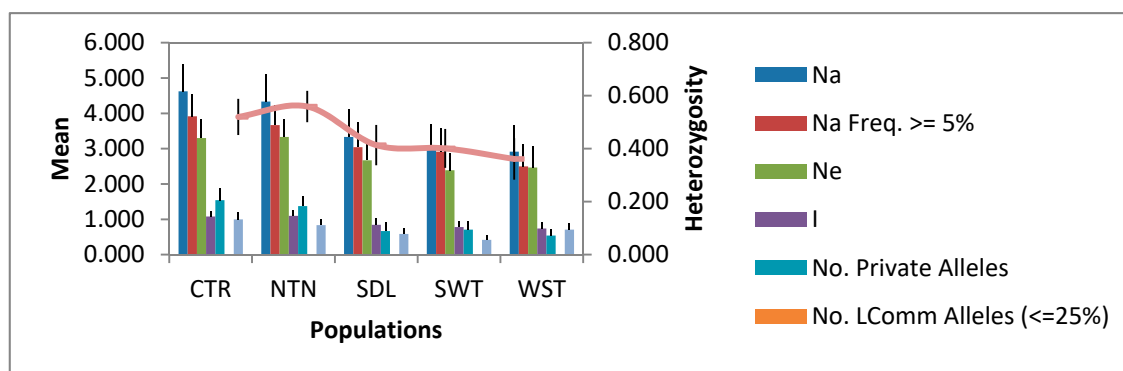

Figure 2. Allelic patterns across the study populations of Brachiaria grass ecotypes in Uganda. CTR = central, NTN = northern, SDL = southern dryland, SWT = southwestern, and WST = western. Na = number of different alleles, Ne = number of effective alleles, and I = Shannon information index.

The Pearson correlation coefficient analysis showed a strongly positive linear relationship between the number of ecotypes in the regional populations and the number of different alleles (r = 0.972; p = 0.006). Similar holds between the sample size and the number of private alleles (r = 0.920; p = 0.027). However, the linear relationships of altitude and rainfall with both Na and Np were negative and non-significant.

3.3. Similarity-Based Analysis

Neighbor-joining (NJ) dendrogram illustrates the genetic relationship among ecotypes as well as between ecotypes and commercial Brachiaria cultivars (Figure 3). The NJ tree constructed based on the genetic distances showed 99 ecotypes and six commercial cultivars in three major groups. Group 1 had two ecotypes, Group 2 had 20 ecotypes and a commercial cultivar *B. humidicola* cv. Humidicola (46 K), and Group 3 had 77 ecotypes and five commercial cultivars (*B. brizantha* cv. Piata (47 K), *B. brizantha* cv. MG4 (48 K), *Brachiaria* hybrid Mulato II (49 K), *B. humidicola* cv. Llanero (50 K), and *B. decumbens* cv. Basilisk (52 K)). Group 3 was further divided into five subgroups with the commercial cultivars present in two subgroups only.
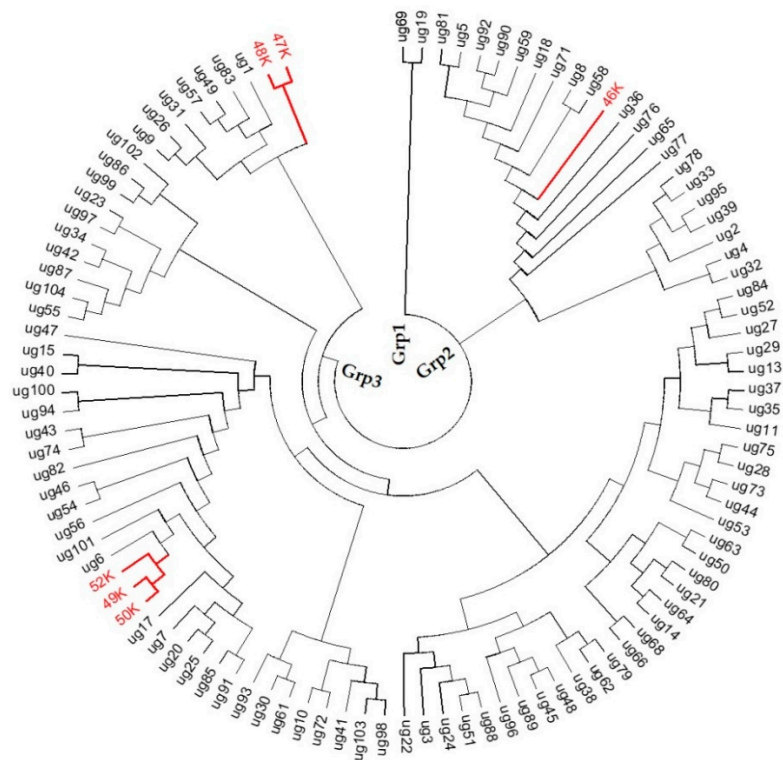
Figure 3. Neighbor-joining tree of 99 *Brachiaria* ecotypes from Uganda and six commercial cultivars based on Rogers's genetic distance calculated from 24 simple sequence repeats markers. Ecotypes are denoted by initial letters ug and commercial cultivars are written in red color as 46 K (*Brachiaria humidicola* cv. Humidicola), 47 K (*Brachiaria brizantha* cv. MG4), 48 K (*Brachiaria brizantha* cv. Piata), 49 K (*Brachiaria* hybrid Mulato II), 50 K (*Brachiaria humidicola* cv. Llanero), and 52 K (*Brachiaria decumbens* cv. Basilisk).

The pairwise Rogers genetic distance for 99 *Brachiaria* ecotypes showed a wide range of genetic differences (≤0.1500 to over ≥0.9001) among the ecotype pairs (Figure 4). About 67.2% of the ecotype pairs had a genetic distance of over 0.9001, 27.6% pairs had a genetic distance between 0.7501 and 0.9000, while 5.2% pairs had a genetic distance of ≤0.7500.
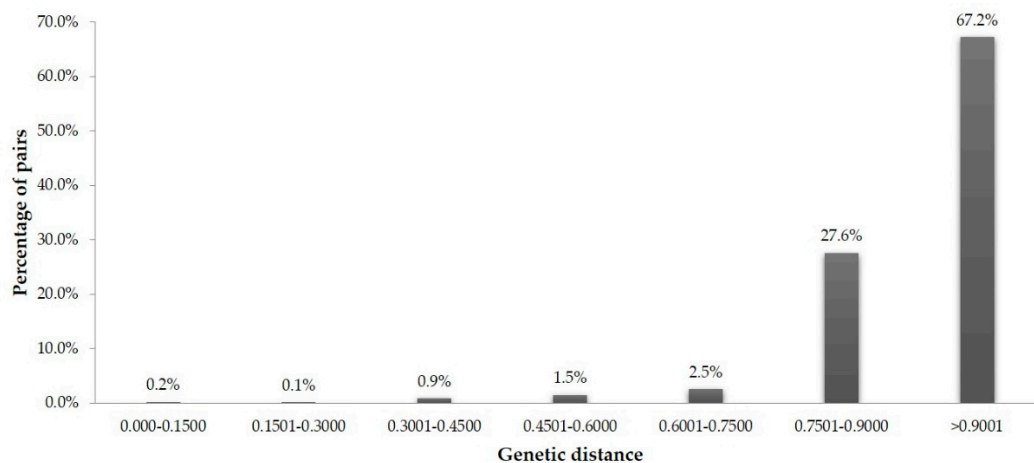

Figure 4. Distribution of pair-wise Roger's genetic distance for 99 *Brachiaria* ecotypes based on 24 simple sequence repeats markers.

3.4. Principal Component Analysis

The genetic relationships among ecotypes were visualized through principal component analysis. Principal component analysis based on allele frequencies generated from 24 SSR markers detected three-major groupings of 99 ecotypes (Figure 5). The percentage variation explained by PC1 and PC2 were 40.6% and 18.2%, respectively.
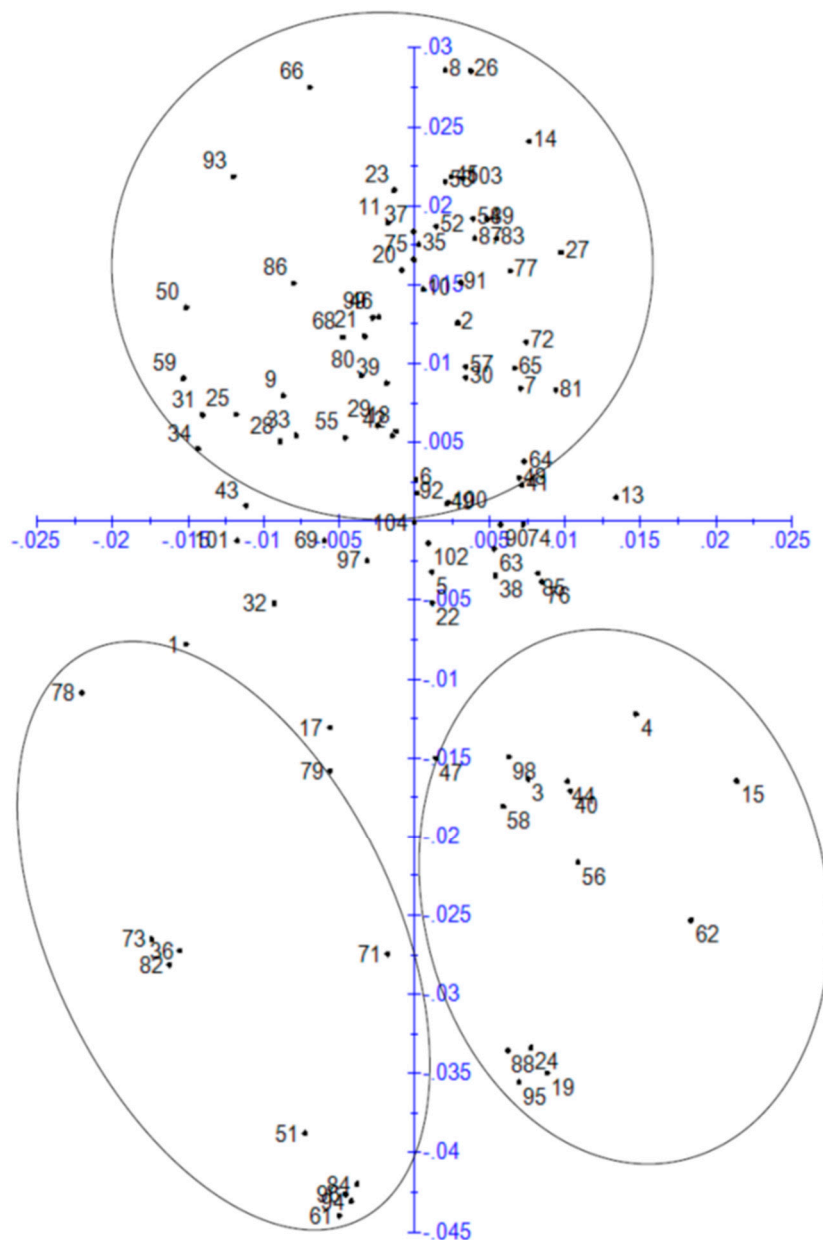

Figure 5. Principal coordinate analysis (PCA) based on genetic distance matrix calculated for 99 *Brachiaria* ecotypes from Uganda based on 24 microsatellite markers. Percentages of variation explained by PC1 and PC2 were 40.6% and 18.2%, respectively.
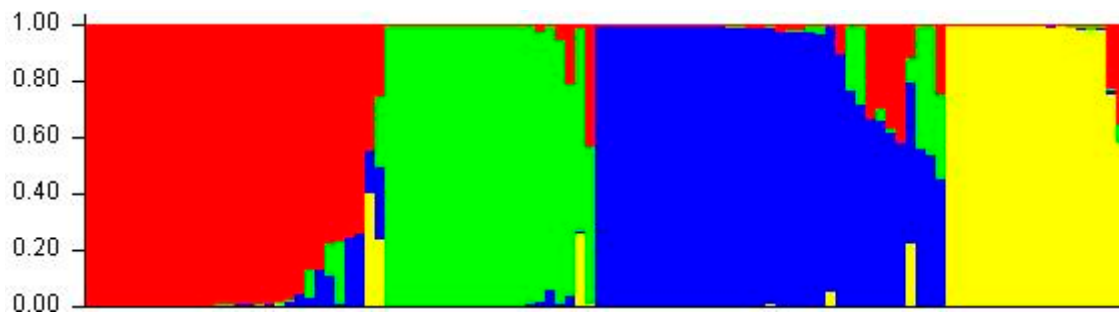
3.5. Structure Analysis

The Bayesian model based clustering algorithm implemented in STRUCTURE software confirmed three distinct clusters (ΔK = 3) among 99 ecotypes (Figure S1). These are indicated in different colors—Cluster I (red), Cluster II (green), and Cluster III (blue). These clusters consisted of pure lines and some admixture individuals with two or three gene pools (Figure 6a). For ΔK = 4, four allelic pools

were identified with four different colors as red, green, blue, and yellow; while ΔK = 6, six allelic pools were identified with six colors as red, green, blue, yellow, purple, and pink. These clusters had pure lines as well as some admixture individuals (Figure 6b,c). As reported in previous study the clustering of ecotypes was independent of their geographical origin [29]. For ΔK = 3, most of the ecotypes from the North, showed the greater probability of ancestral membership (80.5%) for cluster I and II (Table 3).
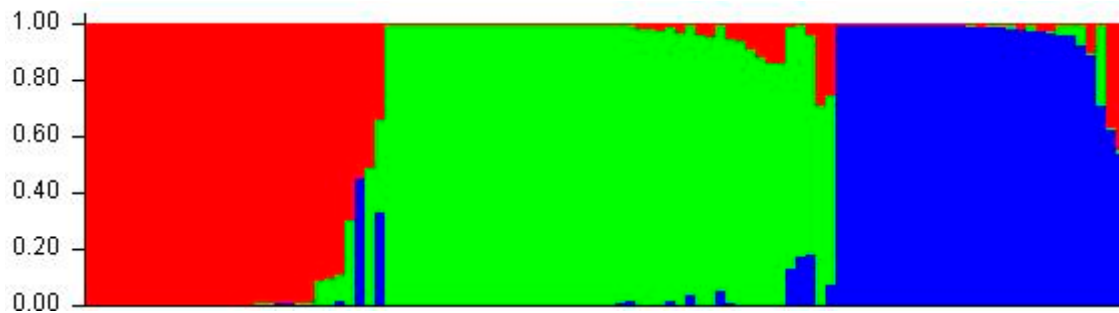
(**a**)

(**b**)
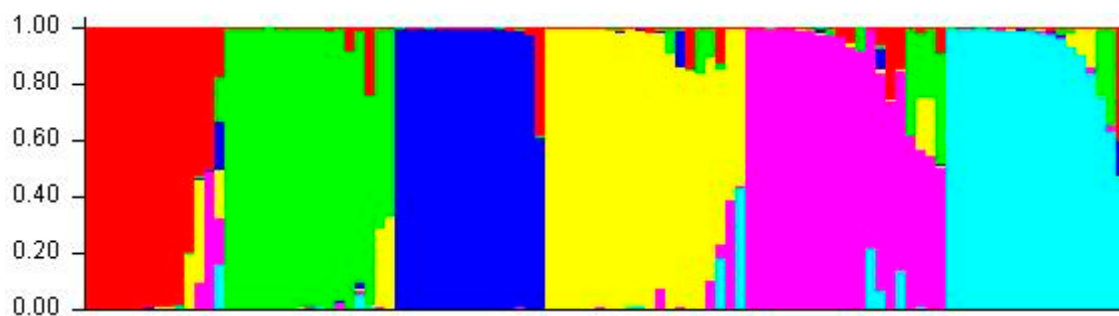
(**c**)

Figure 6. Population structure among individuals with (**a**) ΔK = 3, (**b**) ΔK = 4, and (**c**) ΔK = 6.

Table 3. Proportion of membership of each predefined population from structure analysis (ΔK = 3).

Population	Number of Individual	Estimated Membership Coefficient		
		CI	CII	CIII
Central (CTR)	25	0.384 (10)	0.294 (7)	0.321 (8)
Northern (NTN)	23	0.354 (8)	0.451 (10)	0.194 (4)
South dryland (SDL)	19	0.227 (4)	0.413 (8)	0.361 (7)
Southwestern (SWT)	15	0.284 (4)	0.437 (7)	0.280 (4)
Western (WST)	17	0.186 (3)	0.483 (8)	0.331 (6)

4. Discussion

The genus *Brachiaria* exhibits a great diversity between and within species for genetic composition, morphology, growth habits, adaptation, and agricultural utility. The understanding of the diversity in natural populations is important for genetic conservation as well as for the improvement of a plant species for desirable traits including in *Brachiaria* grass. Of the 100 documented *Brachiaria* species, 33 are represented in the various gene banks, and only seven perennial species of African origin have been explored for forage production [3]. For the past few years, the popularity of improved Brachiaria grass cultivars for pasture production has been increased among livestock farmers in Africa. However, all improved Brachiaria cultivars that are grown in Africa were developed for alien environments in Australia and South America, suggesting a lack of improved Brachiaria cultivars for African environments. This study reports the establishment of the first national collection of *Brachiaria* ecotypes in Uganda, their genetic diversity profiles, and population structure based on SSR markers to facilitate the Brachiaria improvement programs in Uganda. The SSR markers have multiple uses including cultivar identification, genetic diversity studies, and genome mapping [45]. For example, SSR markers have been used to assess genetic diversity in various plant species such as pearl millet, rice, sweet cassava, and *Brachiaria* grass [26,29–31,45,46].

The average polymorphism information content of SSR markers used in this study was 0.89 conferring them as highly informative and capable to differentiate well among the Ugandan *Brachiaria* ecotypes (Table 1). The mean PIC values (0.89) deduced for markers in this study were comparable to studies of Silva et al. for the top 30 most informative markers [21], Kuwi et al. [30], and Pessoa-Filho et al. [47] although it was higher than those reported in other studies [31,48,49]. Interestingly, the number of SSR alleles detected in this study (n = 584) was higher than those reported by Vigna et al. [49], Jungmann et al. [1], and Pessoa-Filho et al. [47], but was lower than in the study of Trivino et al. [50]. Differences in PIC values and SSR alleles among these studies could be attributed to several factors such as differences in number, genetic background, and genetic complexity of *Brachiaria* genotypes; variation in numbers and types of markers used in the analysis, and the difference among studies in allele scoring system and combinations thereof. A relatively higher number of alleles detected in this study may have been associated with geographical position of Uganda in the region where Africa's seven distinct biogeographic regions or phytochoria converge [51] and the region also represents the center of diversity of the genus *Brachiaria* [3]. Besides the robustness of markers in detecting a high number of alleles, there were some challenges in alleles scoring, especially in differentiating stutter and true peaks as reported by other authors [52,53].

The *Brachiaria* ecotypes analyzed in this study were collected from central, northern, south dryland, southwestern, and western regions that represent eight of eleven agroecological zones in Uganda [25]. The majority of these ecotypes (n = 95) were collected from sites with an altitude range of 1080–1521 m above sea level and an annual rainfall of between 1000 and 1500 mm. Despite differences among the collection sites for altitude and amount of precipitation, differences in the allelic patterns (e.g., number of effective alleles and private alleles) in the regional population were mainly influenced by sample size (data not shown) as reported in previous studies [30,54]. The detection of private alleles in all five regional populations suggests them as a valuable sources of genetic variation for breeding programs [55] targeting adaptation and other traits.

Analysis of molecular variance showed a high contribution of within-population difference to the total variation inferring high genetic diversity among the ecotypes. This result is substantiated by a low level of genetic variations among the populations, a high pair-wise Roger's genetic distance of most ecotype pairs, and a fair representation of ecotype from all regions in structure analysis clusters particularly in ΔK = 3. Such differences among the ecotypes is anticipated due to the apomictic mode of reproduction in favor of maternal genotype regardless of the level of heterozygosity [56] and the polyploidy nature of *Brachiaria* species that's often associated with meiotic anomalies leading to reduced pollen fertility [57]. Many Brachiaria species are known to have variable ploidy levels, for example, presence of tetra-, penta- and hexaploidy in *B. brizantha* population [58]. Polyploidy benefits plants from heterosis, gene redundancy, and loss of self-incompatibility and gain of asexual reproduction [59]. Our results were similar to studies of Pessoa-Filho et al. [47] and Vigna et al. [49] on *Brachiaira ruziziensis* and *Brachiaria brizantha*, respectively. The partitioning of molecular variations for the Ugandan ecotype population was similar to those reported in other studies [30,31,60].

The STRUCTURE analysis showed the presence of three distinct gene pools in Ugandan *Brachiaria* ecotypes. The three gene pools detected in this study agrees to previous studies in Tanzania, Ethiopia, and Brazil [30,31,49]. In agreement with STRUCTURE analysis, the NJ tree showed Ugandan ecotypes and six commercial cultivars in three distinct groups, but the membership coefficient to each group differed between two analyses. We observed a high degree of relatedness between ecotypes and the commercial cultivars. Ecotypes in groups 1, and subgroups of groups 2 and 3 that clustered exclusive of improved cultivars may require further analysis to know where they belong, they could possess unique traits of agricultural importance. Such grouping and sub-grouping of ecotypes and improved cultivars in NJ trees signifies a high level of genetic diversity in Ugandan ecotypes compared to six improved *Brachiaria* cultivars that belong to three species (*B. brizantha*, *B. decumbens,* and *B. humidicola*) and a hybrid of *B. brizantha* × *B. decumbens* × *B. ruziziensis*. These observations were anticipated as since many *Brachiaria* species occur naturally in eastern Africa and the region represents a center of diversity of the genus [3]. We also guess that this collection of 99 ecotypes may have representation of several *Brachiaria* species.

Most improved *Brachiaria* cultivars that are in use for pasture production were derived from the direct selection of naturally occurring genotypes from the East Africa [9]. Therefore, the evaluations of these Ugandan *Brachiaria* ecotypes for major agricultural traits, e.g., biomass yield, animal nutrition, livestock productivity, pests and disease resistance, and adaptation to drought at different agroclimatic zones is necessary to develop locally adapted and improved cultivars for commercial cultivation. The SSR markers revealed a high genetic diversity in Ugandan *Brachiaria* ecotypes and their high value in *Brachiaria* improvement programs. Crosses of distantly related ecotypes could be a good strategy to broaden the genetic base. High density genotyping and association mapping would help to shorten the time necessary for completing a breeding cycle and developing new varieties. The complexity of the Brachiaria genome, limited understanding of reproductive biology, and morphological agility within and between the species have limited the pace of *Brachiaria* breeding. Therefore, there is a need to enrich the current understanding of Brachiaria biology and promote integrated use of conventional and molecular breeding methods for better exploitation of genetic resources from this collection as well as those available elsewhere.

5. Conclusions

Through this study, we successfully established the first nation-wide collection of *Brachiaria* ecotypes in Uganda covering five regions representing eight different agroecological zones in the country. Ecotypes are maintained in the vegetative field gene bank at Tororo, Uganda in the guardianship of NaLIRRI, and these materials can be accessed by other researchers following the Ugandan government's guidelines for accessing genetic resources and benefit sharing. This study documented genetic diversity and population structure of these ecotypes using SSR markers. Ecotypes were rich in allelic diversity, genetically diverse and they had three distinct gene pools. High contribution of within ecotypes genetic

difference to total diversity observed in these ecotypes was consistent with the reproductive mode, dispersal mechanism, and genetic attributes of the *Brachiaria* species. The genetic materials (ecotypes) and genetic information produced in this study will form a basis for *Brachiaria* grass conservation and improvement programs targeting agricultural and environmental applications in Uganda and beyond.

References

1.　Jungmann, L.; Vigna, B.B.Z.; Paiva, J.; Sousa, A.C.B.; do Valle, C.B.; Laborda, P.R.; Zucchi, M.I.; de Souza, A.P. Development of microsatellite markers for *Brachiaria humidicola* (Rendle) Schweik. *Conserv. Genet. Res.* **2009**, *1*, 475. [CrossRef]

2.　Renvoize, S.A.; Clay, W.D.; Kabuye, C.H.S. Morphology, taxonomy and natural distribution of *Brachiaria* (Trin.) Griesb. In *Brachiaria: Biology, Agronomy and Improvement*; Miles, J.W., Maass, B.L., do Valle, C.B., Eds.; International Center for Tropical Agriculture: Cali, Colombia, 1996; Volume 1, pp. 1–15.

3.　Keller-Grein, G.; Maass, B.L.; Hanson, J. Natural variation in *Brachiaria* and existing germplasm collections. In *Brachiaria: Biology, Agronomy and Improvement*; Miles, J.W., Maass, B.L., do Valle, C.B., Eds.; International Center for Tropical Agriculture: Cali, Colombia, 1996; Volume 1, pp. 16–42.

4.　Barnard, C. *Herbage Plant Species, Australia Herbage Plant Registration Authority*; CSIRO: Canberra, Australia, 1969; Volume 154.

5.　Oram, R.N. Register of Australian herbage plant cultivars. In *Australian Herbage Plant Registration Authority, Division of Plant Industry*; CSIRO: East Melbourne, Australia, 1990; Volume 304.

6.　Parsons, J.J. Spread of African pasture grasses to the American tropics. *J. Range Manag.* **1972**, *25*, 12–17. [CrossRef]

7.　Argel, P.J.; Keller-Grein, G. Regional experience with *Brachiaria*: Tropical America—Humid lowlands. In *Brachiaria: Biology, Agronomy and Improvement*; Miles, J.W., Maass, B.L., do Valle, C.B., Eds.; International Center for Tropical Agriculture: Cali, Colombia, 1996; Volume 1, pp. 205–224.

8.　Fisher, M.J.; Kerridge, P.C. The agronomy and physiology of *Brachiaria* species. In *Brachiaria: Biology, Agronomy and Improvement*; Miles, J.W., Maass, B.L., do Valle, C.B., Eds.; International Center for Tropical Agriculture: Cali, Colombia, 1996; Volume 1, pp. 43–52.

9.　Jank, L.; Barrios, S.C.; do Valle, C.B.; Simeão, R.M.; Alves, G.F. The value of improved pastures to Brazilian beef production. *Crop Pasture Sci.* **2014**, *65*, 1132–1137. [CrossRef]

10.　Arango, J.; Moreta, D.; Nunez, J.; Hartmann, K.; Dominguez, M.; Ishitani, M.; Miles, J.; Subbarao, G.; Peters, M.; Rao, I. Developing methods to evaluate phenotypic variability in biological nitrification inhibition (BNI) capacity of *Brachiaria* grasses. *Trop. Grassl.* **2014**, *2*, 6–8. [CrossRef]

11.　Moreta, D.; Arango, J.; Soleto, M.; Vergara, D.; Rincón, A.; Ishitani, M.; Castro, A.; Miles, J.; Peters, M.; Tohme, J.; et al. Biological nitrification inhibition (BNI) in *Brachiaria* pastures: A novel strategy to improve eco-efficiency of crop- livestock systems and to mitigate climate change. *Trop. Grassl.* **2014**, *2*, 88–91. [CrossRef]

12. Ghimire, S.; Njarui, D.; Mutimura, M.; Cardoso, J.; Johnson, L.; Gichangi, E.; Teasdale, S.; Odokonyero, K.; Caradus, J.; Rao, I.; et al. Climate-smart *Brachiaria* for improving livestock production in East Africa: Emerging opportunities. In *Sustainable Use of Grassland Resources for Forage Production, Biodiversity and Environmental Protection, Proceedings of 23rd International Grassland Congress, New Delhi, India, 20–24 November 2015*; Vijay, D., Srivastava, M., Gupta, C., Malaviya, D., Roy, M., Mahanta, S., Singh, J., Maity, A., Ghos, P., Eds.; Range Management of Society of India: Jhansi, India, 2015; pp. 361–370.

13. Subbarao, G.V.; Nakahara, K.; Hurtado, M.P.; Ono, H.; Moreta, D.E.; Salcedo, A.F.; Yoshihashi, A.T.; Ishikawa, T.; Ishitani, M.; Ohnishi-Kameyama, M.; et al. Evidence for Biological nitrification inhibition in *Brachiaria* pastures. *Proc. Nat. Acad. Sci. USA* **2009**, *106*, 17302–17307. [CrossRef]

14. Djikeng, A.; Rao, I.; Njarui, D.; Mutimura, M.; Caradus, J.; Ghimire, S.R.; Linda, J.; Ahonsi, M.; Kelemu, S. Climate-Smart *Brachiaria* grasses for improving livestock production in East Africa. *Trop. Grassl.* **2014**, *2*, 38–39. [CrossRef]

15. Bogdan, A.V. *Tropical Pastures and Fodder Plants*; Longman: London, UK, 1977; Volume 475.

16. Sotomayor-Ríos, A.; Vélez-Fortuño, J.; Woodbury, R.; Schertz, K.F.; Sierra-Bracero, A. Description and cytology of a form of Signal grass (*Brachiaria brizantha* Stapf.) and its agronomic behavior compared to Guinea grass (*Panicum maximum* Jack.). *J. Agric. Univ. P R.* **1960**, *44*, 208–220. Available online: https://revistas.upr.edu/index.php/jaupr/article/view/13809 (accessed on 29 September 2019).

17. Vendramini, J.; Seller, B.; Sullenberger, L.E.; Silveira, M. Mulato II (*Brachiaria* sp.). Available online: https://edis.ifas.ufl.edu/ag310 (accessed on 29 September 2019).

18. Ndikumana, J.; de Leeuw, P.N. Regional experience with *Brachiaria*: Sub-Saharan Africa. In *Brachiaria: Biology, Agronomy and Improvement*; Miles, J.W., Maass, B.L., do Valle, C.B., Eds.; International Center for Tropical Agriculture: Cali, Colombia, 1996; Volume 1, pp. 247–257.

19. Miles, J.W.; Maass, B.L.; do Valle, C.B. *Brachiaria: Biology, Agronomy and Improvement*; International Center for Tropical Agriculture: Cali, Colombia, 1996; Volume 288.

20. Jungmann, L.; Sousa, A.C.B.; Paiva, J.; Francisco, P.M.; Vigna, B.B.Z.; do Valle, C.B.; Zucchi, M.; de Souza, A.P. Isolation and characterization of microsatellite markers for *Brachiaria brizantha* (Hochst. ex A. Rich.) Stap. *Conserv. Genet.* **2009**, *10*, 1873–1876. [CrossRef]

21. Silva, P.; Martins, A.; Gouvea, E.; Marco, P.; Ferreira, M. Development and validation of microsatellite markers for *Brachiaria ruziziensis* obtained by partial genome assembly of Illumina single end reads. *BMC Genomics* **2013**, *14*, 17. [CrossRef]

22. Maass, B.L.; Midega, C.A.O.; Mutimura, M.; Rahetlah, V.B.; Salgado, P.; Kabirizi, J.M.; Khan, Z.R.; Ghimire, S.R.; Rao, I. Home coming of *Brachiaria*: Improved hybrids prove useful for African Animal Agricultural. *East Afr. Agric. For. J.* **2015**, *81*, 71–78. [CrossRef]

23. Njarui, D.M.G.; Gichangi, E.M.; Ghimire, S.R.; Muinga, R.W. Climate smart *Brachiaria* grasses for improving livestock production in East Africa—Kenya experience. In Proceedings of the End of the Project Workshop, Naivasha, Kenya, 14–15 September 2016; Kenya Agricultural and Livestock Research Organization: Nairobi, Kenya, 2016; Volume 271.

24. USAID. Uganda Biodiversity and Tropical Forest Assessment. Final Report; 2006. Available online: https://www.vub.be/klimostoolkit/sites/default/files/documents/uganda_biodiversity_assessment_usaid.pdf (accessed on 22 July 2020).

25. FAO. Crop Calendar—An Information Tool for Seed Security. 2010. Available online: http://www.fao.org/agriculture/seed/cropcalendar/welcome.do (accessed on 22 July 2020).

26. da Costa, T.R.; Filho, P.S.V.; Gonçalves-Vidigal, M.C.; Galvan, M.Z.; Lacanallo, G.F.; da Silva, L.I.; Kvitschal, M.V. Genetic diversity and population structure of sweet cassava using simple sequence repeat (SSR) molecular markers. *Afr. J. Biotechnol.* **2013**, *12*, 1040–1048.

27. Garcia, M.; Vigna, B.B.Z.; Sousa, A.C.B.; Jungmann, L.; Cidade, F.W.; Toledo-Silva, G.; Francisco., P.M.; Chiari, L.; Carvalho, M.A.; Karia, C.T.; et al. Molecular genetics variability, population structure and mating system in tropical forages. *Trop. Grassl. Forrajes* **2013**, *1*, 25–30. [CrossRef]

28. Kawube, G.; Kabirizi, J.; Namazzi, C. Genetic characterization of Alupe Napier grass accessions based on simple sequence repeat (SSR). *Am. J. Exp. Agric.* **2015**, *7*, 205–213.

29. Ondabu, N.; Maina, S.; Kimani, W.; Njarui, D.; Djikeng, A.; Ghimire, S. Molecular characterization of Kenyan *Brachiaria* grass ecotypes with microsatellites (SSR) markers. *Agronomy* **2017**, *7*, 8. [CrossRef]

30. Kuwi, S.O.; Kyalo, M.; Mutai, C.; Mwilawa, A.; Hanson, J.; Djikeng, A.; Ghimire, S.R. Genetic diversity and population structure of Urochloa grass accessions from Tanzania using simple sequence repeat (SSR) markers. *Braz. J. Bot.* **2018**, *41*, 699–709. [CrossRef]

31. Tegegn, A.; Kyalo, M.; Mutai, C.; Hanson, J.; Asefa, G.; Djikeng, A.; Ghimire, S.R. Genetic diversity and population structure of *Brachiaria brizantha* (A. Rich) Stapf accessions from Ethiopia. *Afr. J. Range For. Sci.* **2019**, *36*, 129–133. [CrossRef]

32. Prasantha, V.P.; Chandra, S. *ALS-Binary: A Program for Converting Allele Size in Microsatellite Markers to 0–1 (Binary Data)*. *Biometrics*; ICRISAT: Hyderabad, India, 1997; Available online: http://www.icrisat.org/bt-software-d-als.htm (accessed on 12 May 2015).

33. Pritchard, J.K.; Stephens, M.; Donnelly, P. Inference of population structure from multi-locus genotype data. *Genetics* **2000**, *155*, 945–959.

34. Earl, D.A.; Von Holdt, B.M. Structure harvester: A website and program for visualizing structure output and implementing Evanno method. *Conserv. Genet. Res.* **2012**, *4*, 359–361. [CrossRef]

35. Evanno, G.S.; Goudet, R. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Mol. Ecol.* **2005**, *14*, 2611–2620. [CrossRef]

36. Roger, J.S. Measures of genetic similarity and genetic distance. In *Studies in Genetic VII*; University of Texas: Austin, TX, USA, 1972; Publication 7213; pp. 145–153.

37. Liu, K.; Muse, S.V. PowerMarker: An integrated analysis environment for genetic marker analysis. *Bioinformatics* **2005**, *21*, 2128–2129. [CrossRef] [PubMed]

38. Addinsoft. *XLSTAT Statistical and Data Analysis Solution*; Addinsoft: New York, NY, USA, 2020; Available online: https://www.xlstat.com (accessed on 3 June 2020).

39. Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Kumar, S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **2011**, 2731–2739. [CrossRef] [PubMed]

40. Excoffier, L.; Laval, G.; Schneider, S. Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evol. Bioinform.* **2005**, *1*, 47–50. [CrossRef]

41. Excoffier, L.; Lischer, H.E.L. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Mol. Ecol. Resour.* **2010**, *10*, 564–567. [CrossRef] [PubMed]

42. Peakall, R.; Smouse, P.E. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research—An update. *Bioinformatics* **2012**, *28*, 2537–2539. Available online: https://bioinformatics.oxfordjournals.org/content/28/19/2537 (accessed on 28 May 2018). [CrossRef]

43. Meirmans, P.G. Using the AMOVA framework to estimate a standardized genetics differentiation measure. *Evolution* **2007**, *60*, 2399–2402. [CrossRef]

44. Perrier, X.; Jacquemoud-Collet, J.P. DARwin Software: Dissimilarity Analysis and Representation for Windows (Version 6.0.15). 2006. Available online: http://darwin.cirad.fr/darwin (accessed on 17 April 2020).

45. Kapila, R.K.; Yadav, R.S.; Plaha, P.; Rai, K.N.; Yadav, O.P.; Hash, C.T.; Howarth, C.J. Genetic diversity among pearl millet maintainers using microsatellite markers. *Plant Breed.* **2008**, *127*, 33–37. [CrossRef]

46. Singh, R.K.; Sharma, R.K.; Singh, A.K.; Singh, V.P.; Tiwari, S.P.; Mohapatra, T. Sustainability of mapped sequence tagged microsatellite site markers for establishing distinctness, uniformity and stability in aromatic rice. *Euphytica* **2004**, *135*, 135–143. [CrossRef]

47. Pessoa-Filho, M.; Azevedo, A.L.S.; Sobrinho, F.S.; Gouvea, E.G.; Martins, A.M.; Ferreira, M.E. Genetic diversity and structure of Ruzigrass germplasm collected in Africa and Brazil. *Crop Sci.* **2015**, *55*, 2736–2745. [CrossRef]

48. Sousa, A.C.B.; Carvalho, M.A.; Campos, T.; Sforca, D.A.; Zucchi, M.I.; Jank, L.; Souza, A.P. Molecular diversity, genetic structure and mating system of *Calopogonium mucunoides* Desv. *Genet. Res. Crop. Evol.* **2012**, *59*, 1449–1464. [CrossRef]

49. Vigna, B.B.Z.; Jungmann, L.; Francisco, P.M.; Zucchi, M.I.; do Valle, C.B.; de Souza, A.P. Genetic diversity and population structure of *Brachiaria brizantha* germplasm. *Trop. Plant Biol.* **2011**, *4*, 157–169. [CrossRef]

50. Trivino, N.J.; Perez, J.G.; Recio, M.E.; Ebina, M.; Yamanaka, N.; Tsuruta, S.; Ishitani, M.; Worthington, M. Genetic diversity and population structure of *Brachiaria* species and breeding populations. *Crop Sci.* **2017**, *57*, 2633–2644. [CrossRef]

51. White, F. The vegetation of Africa. In *A Descriptive Memoir to Accompany the UNESCO/AETFAT/UNSO Vegetation Map of Africa*; UNESCO: Paris, France, 1983.

52. DeWoody, J.; Nason, J.D.; Hipkins, V.D. Mitigating scoring errors in microsatellite data from wild population. *Mol. Ecol. Notes* **2006**, *6*, 951–957. [CrossRef]

53. Palop-Esteban, M.; Segarra-Moragues, J.; Gonzalez-Candelas, F. Polyploid origin, genetic diversity and population structure in the tetraploid sea lavender *Limomnium narbonense* Miller (Plumbaginaceae) from eastern Spain. *Genetica* **2011**, *139*, 1309–1322. [CrossRef] [PubMed]

54. Szpiech, Z.A.; Jakobsson, M.; Rosenberg, N.A. ADZE: A rarefaction approach for counting alleles private to combinations of populations. *Bioinformatics* **2008**, *24*, 2498–2504. [CrossRef]

55. Salema, K.F.M.; Sallam, A. Analysis of population structure and genetic diversity of Egyptian and exotic rice (*Oryza sativa* L.) genotypes. *C. R. Biol.* **2016**, *339*, 1–9. [CrossRef]

56. Ozias-Akins, P.; van Dijk, P.J. Mendelian genetics of apomixes in plants. *Annu. Rev. Genet.* **2007**, *41*, 509–537. [CrossRef]

57. Santo, F.C.; Guyot, R.; do Valle, C.B.; Chiari, L.; Techio, V.H.; Heslop-Harrison, P.; Vanzela, A.L. Chromosome distribution and evolution of abundant retrotransposons in plants: Gypsy elements in diploid and polyploidy *Brachiaria* forage grasses. *Chromosome Res.* **2015**, *23*, 571–582. [CrossRef]

58. Pagliarini, M.S.; do Valle, C.B.; Santos, E.M.; Mendes, D.V.; Bernardo, Z.H.; Mendes-Bonato, A.B.; Silva, N.; Calisto, V. Microsporogenesis in *Brachiaria brizantha* (Poaceae) as a selection tool for breeding. *Genet. Mol. Res.* **2012**, *11*, 1309–1318. [CrossRef]

59. Comai, L. The advantages and disadvantages of being polyploid. *Nat. Rev. Genet.* **2005**, *6*, 836–846. [CrossRef]

60. Teixiera, H.; Rodriguez-Echeverria, S.; Nabais, C. Genetic diversity and differentiation of *Juniperus thurifera* in Spain and Morocco as determined by SSR. *PLoS ONE* **2014**, *9*, e88996. [CrossRef] [PubMed]