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Development, Validation and Characterization of Genic Microsatellite Markers in *Urochloa* Species

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Abstract

Urochloa (syn. *Brachiaria*) cultivars represent 85% of tropical pastures occupying 114 million hectares of cultivated grasslands in Brazil. Despite the commercial importance of the *Urochloa* species, low molecular information is available and is far from saturating the genome. Investigating and obtaining more markers associated to characteristics of difficult and late expression can benefit in accelerating breeding programs of more important species. Aiming to increase the number of molecular markers, genic microsatellite markers were obtained from transcriptome of *U. decumbens* and analyzed for their cross-amplification to *U. brizantha*, *U. humidicola* and *U. ruziziensis*. Genic microsatellite markers were isolated from a transcriptome obtained of *U. decumbens* "Basilisk" roots. Specific primers were designed for one hundred loci, and 32 were polymorphic presenting polymorphism informative content values ranging of 0.12 to 0.81 (mean 0.54). Amplified microsatellite regions yield an average of 4.44 alleles per locus (ranging of 1 to 13). Cross-amplification to *U. brizantha*, *U. humidicola* and *U. ruziziensis* were successfully performed, although the number of the loci transferred varied among them. Multiple Correspondence Analysis revealed three distinct groups separating accessions and species. Four genetic markers presented high potential to distinguish sexual and apomictic accessions of *Urochloa* and must be further investigated. The genic markers identified in this study are the first set of expressed sequence tagged molecular markers for *Urochloa* species.

Keywords

Brachiaria, Signalgrass, Simple Sequence Repeat, Transferability,

Tropical Forage

1. Introduction

The genus *Urochloa* P. Beauv. spp. (syn. *Brachiaria* (Trin.) Griseb. spp.) comprises about 100 species, distributed in the tropical and subtropical regions in both hemispheres. *Urochloa decumbens* (Stapf) R.D.Webster (signalgrass), *U. brizantha* (Stapf) Webster (palisadegrass), *U. humidicola* (Rendle) Morrone & Zuloaga (koroniviagrass) and *U. ruziziensis* (R. Germain & Evrard) Crins (ruzigrass) are the four principal cultivated species in Brazil and were introduced to Brazil in the early 1960s from eastern Africa [1], their major center of origin and diversity. A collection of 430 accessions of 18 species, including the above mentioned, were introduced in 1987 and deposited in germplasm banks of the Empresa Brasileira de Pesquisa Agropecuária—Embrapa (Brazilian Agricultural Research Corporation), aiming at experimental evaluation and use as forage in the new agricultural frontier of the Brazilian Cerrados [2]. The new *Urochloa* accessions have revealed a significant variability for productivity, nutritional value, spittlebug resistance and adaptation to acid soils. All characteristics are considered for cultivars release, and the new cultivars have promoted a drastic increase of efficiency in Brazilian livestock production, particularly at low-fertility marginal areas [3]. *Urochloa* cultivars represent 85% of tropical pastures occupying 114 million hectares of cultivated grasslands in Brazil [4].

Aforementioned species are represented by accessions with apomictic and sexual mode of reproduction, and also by diploid and polyploid individuals [5]. Furthermore, *U. brizantha*, *U. decumbens* and *U. ruziziensis* form an agamic complex [6], meaning they intercross and produce fertile progeny. Based on this, the International Center for Tropical Agriculture (CIAT) and the Empresa Brasileira de Pesquisa Agropecuária (Embrapa) have conducted breeding programs intraspecific and performing interspecific crosses, both based in recurrent selection methods [3] [4].

New cultivars of tropical forages that better comply with the demands of the livestock sector have influenced and stimulated the conduction of breeding programs for the most important commercial species [1]. New tools to facilitate the selection process such as the use of molecular markers (e.g. microsatellites or Simple Sequence Repeats—SSR) associated to characteristics of difficult and later expression have been studied and searched in order to accelerate breeding and resulting genetic gains [7].

Despite the commercial importance of the *Urochloa* species for tropical grasslands, molecular information has been carried out but still stands far of saturating the genome with markers. Five-hundred primer pairs of SSR markers were obtained for *U. ruziziensis* diploid using a *de novo* partial genome assembly of single-end Illumina reads and were partially transferable to other cultivars

of *Urochloa* [8]. Ninety-three SSR markers were developed for *U. decumbens* from a microsatellite-enriched library [7], of which >98% cross-amplified in *U. ruziziensis* and *U. brizantha* accessions. Worthington *et al.* [9] obtained 706 single nucleotide polymorphism (SNP) markers heterozygous using genotyping by sequence (GBS) in an interspecific progeny of *U. decumbens* X *U. ruziziensis*. Considering these species genomes are average-sized 1633 Mbp·C^{-1} and 1230 Mbp·C^{-1} [10], respectively, this means one marker per 1.0 Mbp, approximately. Therefore, more markers are necessary to perform genetic studies such as saturated linkage mapping, marker-associated and marker-based selection.

Expressed sequence tags—derived simple sequence repeat markers (EST-SSRs) have a number of advantages over genomic SSR markers developed by cloning and sequencing [11]. In *Urochloa* genus, and particularly in the agamic complex of *U. brizantha*, *U. decumbens* and *U. ruziziensis*, the genic SSR markers are expected to be more transferable and more likely to be associated with characteristics of interest in breeding programs.

The objectives of this study were: 1) to investigate the polymorphism level and occurrence of genic microsatellite obtained from root transcriptome in *U. decumbens*, and 2) to analyze the transferability of genic SSR markers across *Urochloa* species.

2. Material and Methods

2.1. Plant Materials

Urochloa decumbens transcriptome was the base for development, validation and characterization of the genic microsatellite markers. Seven *U. decumbens*, including apomictic and sexual accessions were used to characterize the SSR genic markers. Four accessions of *U. brizantha*, three of *U. ruziziensis* and one of *U. humidicola* were tested for cross-amplification. The accessions identification, as recorded in the databases of Embrapa Gado de Corte and Center for Tropical Agriculture-CIAT, their mode of reproduction, origin and ploidy are shown in **Table 1**.

2.2. Genic SSR Development

The transcriptome from *U. decumbens* roots under aluminum stress was obtained to support studies about genetic expression, as described by Salgado *et al.* [12], and was used to develop transcriptome-derived microsatellite markers.

The searches for genic SSR markers from the contig dataset were performed using MISA (Microsatellite Identification Tool) [13]. The definition of microsatellites was set to 12-fold (1/12), di (2/6), tri (3/5), tetra (4/5), penta (5/5), or hexa (6/4) mononucleotide repeats. After screening, genic SSR markers situated more than 150 bp from both ends of the encompassing unigene were retained and used to design primers with Primer 3 [14]. For this study, mono and dinucleotide genic SSR were excluded, once they would not be suitable for further studies using the available capillary electrophoresis system.

Table 1. Species and accessions of *Urochloa* used to characterize microsatellite markers and analyze cross-amplification.

Species	EGC	BRA	CIAT	Origin	MR	Ploidy	Cultivar	Representative character
U. decumbens	D06	004456	16,495	Kenya	SEX	2x	–	–
U. decumbens	D07	004472	16,497	Kenya	APO	4x	–	–
U. decumbens	D24	004651	26,295	Rwanda	SEX	2x	–	–
U. decumbens	D35	004782	26,308	Rwanda	SEX	2x	–	–
U. decumbens	D40	004766	26,306	Rwanda	SEX	2x	–	–
U. decumbens	D61	001961	16,100	–	APO	4x	–	–
U. decumbens	D62	001058	606	Uganda	APO	4x	"Basilisk"	Aluminum tolerance
U. brizantha	B30	000591	6294	Kenya	APO	4x	"Marandu"	Spittlebug resistance
U. brizantha	B112	002844	16,125	Ethiopia	APO	4x	"BRS Piatã"	Early flowering and high seed production
U. brizantha	B166	003891	16,467	Kenya	APO	4x	"BRS Paiaguás"	High yield on dry season
U. brizantha	B178	004308	26,110	Burundi	APO	5x	"Xaraés"	Spittlebug resistance
U. humidicola	H16	005118	26,149	Burundi	APO	4x	"BRS Tupi"	Waterlogging tolerance
U. ruziziensis	R46*	–	–	–	SEX	4x	–	Higher nutritional quality and *in vitro* digestibility of dry matter
U. ruziziensis	R48*	–	–	–	SEX	4x	–	
U. ruziziensis	R50*	–	–	–	SEX	4x	–	

Note: EGC codes from Embrapa Gado de Corte, BRA codes from Embrapa, CIAT codes from Center for Tropical Agriculture, MR mode of reproduction—sexual or apomictic, – not available, *artificially tetraploidized genotypes.

Aiming to select molecular markers with the minimum redundancy in *Urochloa*'s genomes, the predicted amplified sequences were submitted to BLASTn against *Setaria italica* genome using the online platform CoGe: Blast [15] with an e-value threshold of 1E-10. Sequences returning one or two hits were retained and blast matches positions, along *S. italica* genome, were considered to select 100 genic SSR markers distributed all over genome. *S. italica* genome was chosen as model since it is the most similar genome available in public databases [12].

2.3. DNA Isolation and Microsatellite Genotyping

Genomic DNA was isolated from young fresh leaves using the CTAB method [16]. The purity and concentration of the isolated DNA were determined using a NanoDrop 1000 (Thermo Scientific) spectrophotometer and by electrophoresis in a 0.8% agarose gel that was subsequently stained with ethidium bromide (5 $\mu g/mL^{-1}$). The polymerase chain reaction (PCR) assays were performed in a MJ Research PTC-100^{TM} thermocycler in a 25 µL final volume of buffer [20 mM Tris–HCl (pH 8.4), 50 mM KCl and 1.5 mM MgCl] containing 60 ng of genomic DNA, 0.8 µM of each primer (forward and reverse), 150 µM dNTPs and 1 U Taq DNA polymerase (Invitrogen). The PCR program consisted of an initial denaturing step at 94˚C for 5 min followed by 30 cycles of amplification [94˚C (1 min), "X"˚C (1 min), 72˚C (1 min)] and a final elongation step at 72˚C for 15 min, being "X" the temperature determined to amplify each locus (**Table 2**). The

Table 2. Description of 34 genic microsatellites developed for *U. decumbens* based on a root transcriptome.

Locus	Primer sequences (5'–3')	Repeat motif	Ta (°C)[a]	Size (bp)	Total No. alleles	*U. brizantha*	*U. decumbens* diploid	*U. decumbens* tetraploid	*U. humidicola*	*U. ruziziensis*	PIC
						No. alleles	No. alleles	No. alleles	No. alleles	No. alleles	
DecSSR181	F: AGTTGGTGTTGGCAATAACTCTG R: CTTCTTGGCAGTTGAAGCATCT	CGG(3*5)	60	180 - 210	5	4	1	2	2	3	0.60
DecSSR184	F: CCGAACTTGAGTTGTTATTTCCA R: AACTGATGGTAACTCTTGGAGCA	TGG(3*5)	60	120 - 140	2	1	1	1	2	1	0.12
DecSSR186	F: CAGGAACGGGGAGACGAG R: CTCACCAACGTCGGCCTC	CGG(3*5)	60	80 - 90	1	1	1	1	x[b]	1	x
DecSSR187	F: GGTGCGTACAACAACTACTGATCT R: CTGTGCACGCTCGCTGAC	GCT(3*5)	62	150 - 170	5	2	5	2	x	2	0.66
DecSSR188	F: GTGTGCCTCTGAGACCCAGT R: CCCAGCTCAGCCAGTACTTC	GGA (3*5)	62	120 - 140	4	1	1	3	1	1	0.31
DecSSR194	F: CTCCGCCTTGCAGTACCTC R: TGCAGTACAACAACTACCACCTC	GCT(3*5)	60	110 - 120	2	2	2	2	x	2	0.50
DecSSR195	F: GTCCCGGTGGTGGTGTCC R: GTTCCACCTGATCTCGCTCTC	GGA(3*5)	62	90 - 100	4	2	1	3	x	2	0.56
DecSSR199	F: ATTCAGATGCATTTTTCTTCGTC R: AGCAGCGTATAATAAGCAAGCAC	TCA(3*5)	60	140 - 160	2	x	2	2	x	2	0.46
DecSSR202	F: CTGCTTCTTATAGATCCGACCAC R: TCTTTAGGAGAGGGATCGAGATT	TCG(3*5)	60	110 - 150	7	1	4	2	1	4	0.81
DecSSR203	F: GTGATGACGGATGCGGTT R: ACTATCCGACTTCGCCCAC	GCG(3*5)	62	110 - 100	4	3	2	4	x	1	0.66
DecSSR205	F: GAAATCAGAGATGCCCAGACC R: AGCGAGATCACCACGGAG	CCG(3*5)	62	90 - 110	2	1	2	1	x	1	0.14
DecSSR206	F: GGTGGTACCCGGAGTTAGAGTT R: GCTACCACTACGACCAGGACTC	GCC (3*5)	62	100 - 110	3	1	1	2	1	1	0.24
DecSSR211	F: GTATTAATTCACTGGGTGTCGCT R: CCATCACCTCCATCATAGGC	TTG(3*5)	60	130 - 150	4	1	1	1	2	2	0.56
DecSSR212	F: GCCGTATCTCCTTCTCCCAC R: CACTACCCCTCCCTCCTCTC	GAG(3*5)	62	110 - 140	13	11	5	5	3	4	0.84
DecSSR213	F: CAGAGGAAAGGAAGCAGCAG R: GGCATCACCACCGTCTGG	CAG(3*5)	62	110 - 130	8	7	2	4	x	3	0.81
DecSSR214	F: AGGAACTGGTGCTCCTTCTTC R: TCCTACAAGTACACAATCCCACC	GGC(3*5)	60	120 - 160	7	5	3	5	1	5	0.77
DecSSR217	F: TCTGCAAACTGAGGTTCTTCTTC R: TTGCCAAACGAGACCATAGAGTA	TTC (3*5)	55	260 - 310	4	1	1	3	x	1	0.41
DecSSR218	F: TATCTCTATCTGGGGATTGGAGC R: AAACAAAACCAAATCGCGG	GCG(3*6)	60	140 - 160	6	3	3	4	3	2	0.80
DecSSR221	F: ATCGACACTACTCTCGGCATAAC R: GTATGTGGTGGAGGTAGTTGGG	TCG(3*5)	60	120-140	3	1	1	1	2	2	0.55
DecSSR222	F: GGTCATCACCGACTGGTTATTTA R: ACTTGACAAATCTCTGTCCCGTA	GGC(3*5)	55	130 - 140	5	4	1	3	2	4	0.63
DecSSR224	F: AAGACGAAGCGAGCGTTG R: CTTCCTCTCCTGGTCGTCCT	CGG(3*5)	62	90 - 100	2	1	2	1	1	2	0.33
DecSSR228	F: GTCGTCGAGCTTCCCGAG R: CTGCTAGAGACGCGGAGC	GCC(3*5)	60	100 - 110	3	2	1	2	1	2	0.51

Continued

DecSSR235	F: CATGTGCAAGATCTACGTGTCC R: ATTCACGGAGAGATTAGGCTCC	GAA(3*5)	60	130 - 140	3	1	3	1	x	2	0.32
DecSSR238	F: AGTATGGCTCCTTCAGTCACAAT R: GGTGGTTCATGAGAGGAGGA	CAG(3*5)	55	110 - 120	3	1	1	2	2	2	0.54
DecSSR239	F: GTACCCTTACGACAGCCACC R: CAAATCCATTTTTGTCAGTGGTT	CAG(3*5)	62	130 - 150	3	1	3	2	x	2	0.53
DecSSR246	F: CGTCTTGGAGGAGGATGAAG R: GGACCTGGATGTCCGCAT	GGA(3*5)	62	110 - 130	8	6	3	5	2	4	0.80
DecSSR250	F: GGTGTTTGGTGGTGCACAG R: CTGGCCCTCCCTCTCCCT	GAG(3*5)	62	80 - 100	6	4	2	4	x	5	0.79
DecSSR251	F: GAATTAACATCATCAGGTCAGGC R: AGATGGTGCTCGTATGGAAGTC	GTT(3*5)	62	120 - 150	6	2	5	2	x	4	0.80
DecSSR254	F: CGACTGAAGAAGAAGGATGTTGA R: CAAAATTTCATGCAGCAAGTACA	TGC(3*5)	60	150 - 160	4	2	1	4	1	4	0.63
DecSSR259	F: CAGCAGTACACTATCCAACATTCA R: ACAAGCTCATGCAAGTTCGTC	GCT(3*6)	60	120 - 150	7	4	2	1	x	4	0.79
DecSSR262	F: AGAAACCTGCTAATGCTTGGAAT R: GTCCATGTGGCTCTCGCT	TCCGAG (6*4)	62	110 - 130	3	2	1	2	1	2	0.53
DecSSR266	F: GAAGTATCTTCTCCCTCTCGTCG R: AAGCAGAAGACGCAGTAAATCC	CTG(3*6)	55	120 - 130	4	4	3	3	x	3	0.72
DecSSR267	F: ACGCCCAACCAACAAGGAC R: TCCAGGAGAGGGTCCTGAG	GGCTCC (6*4)	55	90 - 120	5	3	2	4	x	4	0.70
DecSSR279	F: AGAGCAGAGCTTCTCCTCAGC R: TTAGACTGTGTCGCTACATGACC	GTC(3*5)	62	100 - 110	3	1	1	1	x	1	x
Average						2.71	2.12	2.60	1.64	2.56	0.54

[a]Amplification temperature (˚C), [b]x means no amplified PCR products NCBI deposit ID: 2306544.

amplified products were separated by electrophoresis through 3% agarose gels prior to vertical electrophoresis through 6% denaturing polyacrylamide gels stained with silver according to [17]. The product sizes were determined by comparison to those of a 10 bp DNA ladder (Invitrogen). Only the strongest bands were considered because the less intense bands in the polyacrylamide gels might have been stutter bands.

2.4. Locus Function Annotation

Function annotation of the unigenes containing the microsatellites DecSSR 202, DecSSR 203, DecSSR 218 and DecSSR 221 was conducted by searches against Pfam 31.0 database [18], using the online platform (http://pfam.xfam.org) with default parameters. For identification of probable orthologous genes, the transcript sequences were used as queries for BLASTn searches against Phytozome v12.0 ([19] www.phytozome.net) grass database using the default parameters. Expression profiles of the orthologous genes, from close related species (*Setaria italica*, *Panicum virgatum* and *Sorghum bicolor*), were also obtained in Phytozome v12.0.

2.5. Data Analysis

For cross-amplification analysis, a genic SSR marker was considered transferable when a band of the expected size was amplified via PCR and an appropriate microsatellite pattern was observed.

As conventionally established [20], markers were treated as dominant due to tetraploid level of some accessions; thus, all analyses were based on the presence (1 or yes) or absence (0 or no) of a band in the polyacrylamide gels. The polymorphic informative content (PIC) of each marker was determined according to Anderson *et al.* [20] as follows:

$PIC_i = 1 - \sum_j p_{ij}^2$ where p_{ij} is the frequency of the jth genic marker for accession i and the summation extends over all such patterns. Because of the categorical nature, the set of molecular data was analyzed using Multiple Correspondence Analysis (MCA) to detect structural organization of accessions, with graphical representation through R [21] with "FactoMiner and factoextra" packages.

As an additional means of validation, the genetic distance among accessions was quantified by the Jaccard's coefficient and the analysis was performed based on a binary matrix of molecular data. A rooted tree was constructed using the unweighted pair group method with arithmetic mean (UPGMA). Both analyses were performed in R [21]; we used the "vegan" package for Jaccard's coefficient and, the "cluster and phangorn" packages for UPGMA.

3. Results

3.1. Genic Microsatellite Polymorphism and Transferability

A total of 14,932 microsatellites (excluding mononucleotide repetitions) were identified in 14,822 transcripts, ranging from 12 to 240 nucleotides in length and possessing four to eleven motif repeats. Trinucleotide repeat motifs were the most common microsatellites, accounting for 61% of all identified, and (GCG)*5 and (CGC)*5 were the most frequent.

This set of microsatellites, a hundred genic SSR markers were selected and 34 amplified successfully in *U. decumbens*. Of these, 151 alleles were revealed, leading to a mean number of 4.4 alleles per locus (ranging from 1 for DecSSR186 and DecSSR279 to 13 for DecSSR212). Thirty-two microsatellite loci were polymorphic (86%) among the seven *U. decumbens* accessions. The mean PIC value was 0.54 (range 0.12 - 0.81) (**Table 2**). The most informative loci in this panel of SSR markers were DecSSR202, DecSSR212, DecSSR213, DecSSR218, DecSSR246, DecSSR251 (PIC ≥ 0.8), and the lowest was DecSSR184 (0.12) (**Table 2**). The cross-amplification survey showed 100% of the polymorphic markers amplified PCR products in *U. ruziziensis* tetraploid, 97% in *U. brizantha*, and 46.9 % in *U. humidicola*.

3.2. Multiple Correspondence and Diversity Analysis

The two dimensions of MCA explained approximately 33% of the total variance

(Figure 1). The agamic complex composed by *U. brizantha*, *U. decumbens* and *U. ruziziensis* was closely aligned at crossing point of two axis in the graphic and the *U. humidicola* accession was positioned on the far right of dimension 1 (Dim1, Figure 1). Considering markers with 5% contribution to variance, five alleles of five SSR markers are highlighted on MCA and they were the most important to separate H16 from agamic group, and may contribute to separate sexual and apomictic accessions.

UPGMA dendrogram showed three clusters (Figure 2). One cluster is formed by the three autotetraploid *U. ruziziensis* accessions, all of sexual mode of reproduction, and five *U. decumbens* accessions, in which four are sexual and diploid and one is apomictic and tetraploid (D61). The second cluster is composed by apomictic and tetraploid *U. decumbens* and *U. brizantha* accessions. This cluster can be divided in two subgroups, in which one subgroup contain all four *U. brizantha* accessions and the other subgroup, with the apomictic *U. decumbens* D07 and D62. Lastly, the third cluster contain the *B. humidicola* "BRS Tupi" (H16), the most dissimilar species evaluated.

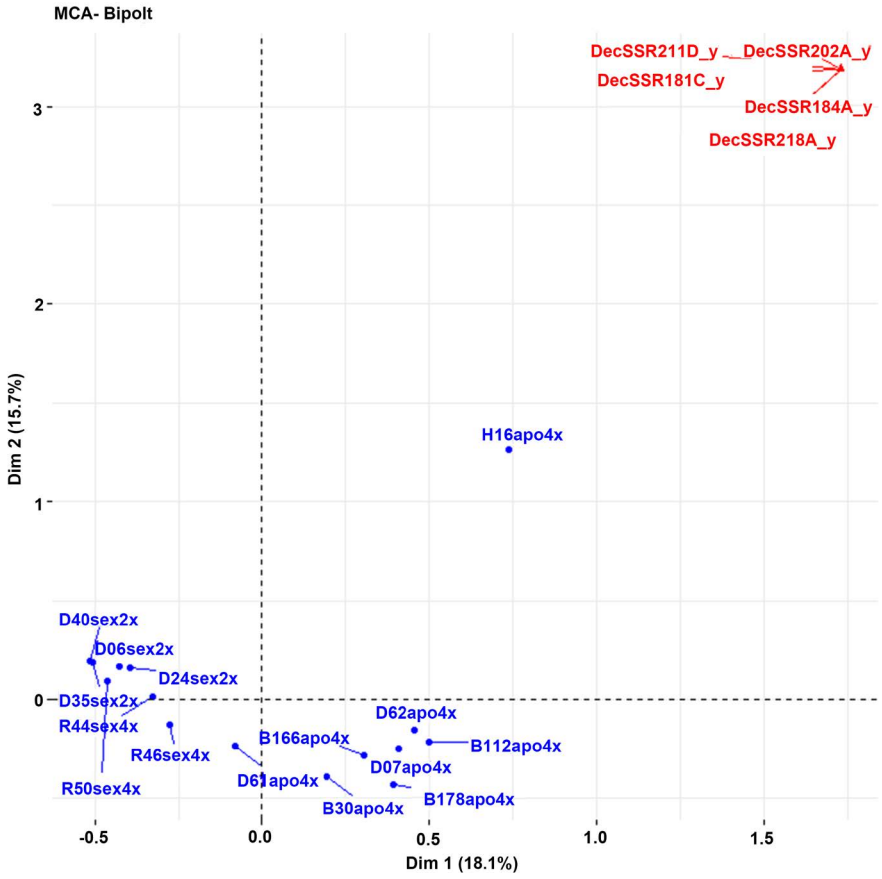

Figure 1. Multiple correspondence analysis of the 15 accessions of *Urochloa* spp. studied. Each accession is coded according ECG numbers (Table 1) followed by mode of reproduction designation (apo—apomictic or sex = sexual) and ploidy level (2x—diploid and 4x—tetraploid). Upper right corner shows the loci presenting more than 5% variance contribution in the first two dimensions (Dim). Axis numbers between parentheses are the proportion of variance explained in each dimension.

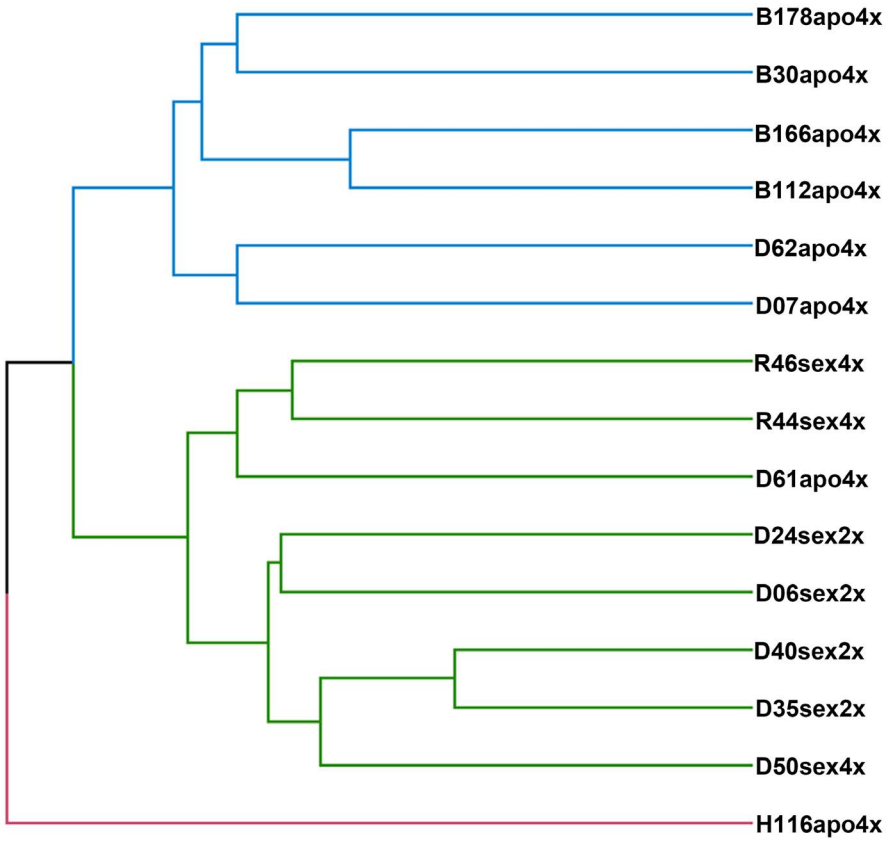

Figure 2. Rooted pair group method with arithmetic mean tree, based on the Jaccard dissimilarity index calculated from EST-SSR data, assembling 15 *Urochloa* accessions belonging to *U. decumbens*, *U. brizantha* and *U. ruziziensis* agamic complex and *U. humidicola*. EGC numbers in **Table 1** identifies accessions, apo or sex identifies the mode of reproduction apomictic or sexual assignments, respectively, and 2x or 4x identifies the accession ploidy level. Comparison between mode of reproduction groups presented significance (p = 8e−4) using multivariate analysis and correcting by step-down sequential correction Bonferroni.

Based on results of MCA and cluster analyses, some genic SSR were further investigated. For DecSSR202 locus, six alleles in *U. decumbens* were observed, four alleles in *U. ruziziensis* and one in *U. brizantha*. The unique allele amplified in apomictic *U. brizantha* accessions was also present in apomictic accessions of *U. decumbens*, but not in sexual ones and not in *U. humidicola*. The other five alleles observed in apomictic *U. decumbens* are also randomly present in sexual accessions. Loci DecSSR203, DecSSR218 and DecSSR221 show four, six and three alleles, respectively, and similarly for DecSSR202, the allele's presence varied individually in frequency between sexual or apomictic accessions. Adding DecSSR184 and DecSSR211, these SSR markers were the more important to divergence between accessions and species (**Figure 2**).

3.3. Functional Annotation

The transcript sequences, containing DecSSR202, DecSSR203, DecSSR218 and

DecSSR221 SSRs were queried against Pfam and Phytozome databases for functional annotation (Table 3) and investigation of their orthologous genes expression patterns. None of these four unigenes presents similarity to the apospory-specific region (ASGR), sequenced by Conner *et al.* [22], nor they are meiosis-specific genes (Table 3). However, expression data for close related species (*Setaria italica*, *Panicum virgatum* and *Sorghum bicolor*) reveal high expression levels of DecSSR203 and DecSSR218 orthologous genes in different stages of panicle development, suggesting these genes could play a role on reproductive development in grasses.

4. Discussion

In the present study, trinucleotide repeat motifs were the most abundant form of microsatellite, consistent with other publications that obtained SSR markers using EST sequencing for different plant species [24] [25] [26]. The method used here has proved to be useful mostly because it is functionally-associated, which provides an effective means to develop molecular markers that target nucleotide diversity in conserved genic regions allowing a high degree of transferability between related species.

Amplification success of genic microsatellites (32%) was lower than evidenced in previous studies using genomic microsatellite-enriched library as source for the development of primers [7] [27] [28] [29] [30]. Moreover, Silva *et al.* [8] obtained 88.9% success rate of PCR amplification for microsatellite loci generated based on partial *de novo* genome assembly of *U. ruziziensis*. Eujayl *et al.* [24] and Chagné *et al.* [32] also evidenced a low rate of amplification of EST-SSR compared with SSR markers isolated from total genomic DNA in wheat and pines, respectively. This lack or low amplification obtained for genic SSR could be explained by the quality of the primer pairs and/or the presence of introns at primer annealing regions. The low mean of alleles per locus (4.4 alleles/locus)

Table 3. Functional annotation of four loci potentially linked to apomixis in *Urochloa* and their probable orthologous loci in *Setaria italica*, *Panicum virgatum* and *Sorghum bicolor*.

SSR locus	Protein name	Pfam domain number	Pfam E-value	Probable orthologous loci in grasses	Blastn E-value
DecSSR202	Homeobox-leucine zipper protein ATHB-54-related	PF00046 PF02183	2.3e−17 4.7e−17	Seita.1G306100 Pavir.Aa00462 Sobic.004G251300	0.00 0.00 0.00
DecSSR203	Protein of unknown function	PF04783 PF04782	6.0e−22 7.6e−96	Seita.5G179100 Pavir.Ea01929 Sobic.003G168100	0.00 0.00 0.00
DecSSR218	Splicing factor 3B subunit 2-like	PF04037 PF04046	7.1e−36 3.4e−21	Seita.4G290900 Pavir.Aa00053 Sobic.010G278800	3.3e−75 3.1e−31 2.0e-65
DecSSR221	tRNA (guanine(37)-N(1))-methyltransferase	PF02475	1.2e−60	Seita.J001500 Pavir.Eb02431 Sobic.007G058001	0.00 0.00 2.0e−166

was also evidenced in this work and corroborates the information reported as a disadvantage of genic SSR as they do tend to show a lower rate of polymorphism (in terms of allelic richness) than those derived from genomic libraries [23] [31] [32] [33] [34].

However, EST-SSR markers exhibit a great advantage over SSR markers developed via cloning and sequencing because they are more widely transferable between species and even genera [11], as confirmed in the present work. Although the proportion of transferability of genic SSR markers to species of the same agamic complex (*U. brizantha* and *U. ruziziensis*) was similar to that obtained for SSR derived from genomic libraries (>90%) [7] [8] [29], the proportion of transferability was higher in *U. humidicola* using genic SSR polymorphic primer pairs (47%) than evidenced using SSR isolated from total genomic DNA (38% [7]).

Interspecific crossings in *Urochloa* breeding seeks to combine main forage characteristics. Moreover, apomictic interspecific hybrids obtained after crosses allow heterosis fixation in a cultivar, which is desirable commercially [9]. Apomictic and sexual individuals are discriminated by embryo sac analysis and/or progeny tests, but both methods are time consuming and expensive. Molecular markers linked to mode of reproduction have been used in order to discriminate apomictic and sexual in offspring at the seedling stage [9] [35]. However, markers have performed inconsistently or have been unable to generate bands linked to apomixis in other crosses, no matter if intraspecific or interspecific. Recently, the primer pair p779/p780 was developed [36] and was linked to the apospory-specific genomic region (ASGR) in *Pennisetum* and *Cenchrus* species. This gene was tested in a diversity panel comprising four apomictic interspecific *Urochloa* hybrid cultivars and accessions (apomictic and sexual) of *U. brizantha*, *U. decumbens*, *U. humidicola* and *Panicum maximum*, and was broadly predictive for apomixis [9]. However, p779/p780 was not efficient as diagnostic marker for apomixis in interspecific hybrids when different apomictic accessions of *U. brizantha* were used as male parent at Embrapa Gado de Corte (L. Chiari, T. G. Déo, unpublished data). Markers associated to apomixis in *Urochloa* species have shown some specificity when they are applied to other species of the genus or applied to other accessions used in crosses, such as p779/p780 and GBS-derived KASP markers [9]; SCAR marker N14 [9] [37] and RAPD marker for apomixis in *U. humidicola* [38]. Those results are comprehensive and expected considering the ASGR has been mapped to a physical large hemizygous region of reduced recombination [39], resulting in inheritance of apospory as a single dominant Mendelian factor. Accordingly, the markers found in recent researches are linked but are not causative of characteristic mode of reproduction. Based on this premise, other associated loci should be revealed and must be studied. In this work, four genic SSR markers may be linked to apomixis in *U. brizantha* and *U. decumbens*. Further studies involving segregating progeny evaluations and markers are still necessary in intra and interspecific crosses in both species,

since population structure may have caused spurious associations leading to false-positive observation [40].

5. Conclusion

The present study described the first effort to develop genic SSR markers based on *Urochloa decumbens* transcriptome. A set of 34 primer pairs have been developed, tested and transferred between *Urochloa* species of different modes of reproduction and ploidy. Overall, above 98% of polymorphic 32 genic SSR markers have been transferred to species of the same agamic complex and 47% to *U. humidicola*, a species with a distinct genomic structure. Our results also reinforce the evidences of broad cross-transferability of genic markers. This study provides new genic markers potentially useful and transferable to other *Urochloa* species that will add molecular information aiming at studies of genetic mapping, genetic association and genomic selection for economically important characteristics in this tropical grass forage.

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