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Esterase polymorphism for genetic diversity analysis of some accessions of a native forage grass, *Mesosetum chaseae* Luces, from the Brazilian Pantanal

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Abstract

The aim of the present study was to estimate the genetic diversity within the samples of *Mesosetum chaseae* from the Embrapa Pantanal Germplasm Bank (BAG) and assess how they are genetically structured to guide proposals to: 1) identify native forages for further testing to measure their suitability for sowing in conjunction with or as an alternative to exotic forages, mainly *Urochloa humidicola*; and 2) improve the species *M. chaseae* with samples that are maintained in the BAG. Isozyme α- and β-esterases were analyzed in 10 accessions collected from different locations in the Nhecolândia sub-region of the Pantanal, and maintained in the BAG. Accessions A11, which showed the highest effective number of alleles, and A32 with the highest average values of expected and observed heterozygosity, were identified as warranting further study as possible options for sowing as pasture forages, as well as for use in recovering poor and degraded areas in the Pantanal region. A high level of population differentiation was detected among the 10 accessions, indicating that they form genetically structured populations and that all accessions are important samples of *M. chaseae*, which should be maintained in the BAG. Crosses between sample plants with the highest genetic distances are recommended to implement improvement plans with a prospect of broadening the genetic base of the species.

Resumen

El objetivo del estudio fue estimar la diversidad genética entre las muestras de *Mesosetum chaseae* del Banco de Germoplasma (BAG) de Embrapa Pantanal y evaluar cómo están estructuradas genéticamente con el objeto de guiar propuestas para: 1) identificar un forraje nativo para estudios de su potencial como especie complementaria o alternativa a forrajes exóticos, sobre todo *Urochloa humidicola*; y 2) mejorar la especie *M. chaseae* con base en muestras disponibles en el BAG Pantanal. En un total de 10 muestras recolectadas en diferentes localidades de la subregión Nhecolândia del Pantanal, se analizaron las isoenzimas α- y ß-esterasas. Se identificaron las accesiones A11, que mostró el número efectivo más alto de alelos; y A32, que presentó los más altos valores promedio de heterocigosidad esperada y observada, como promisorias para trabajos de investigación adicionales con el objeto de desarrollar nuevas opciones tanto para pasturas como para recuperar áreas degradadas en la región del Pantanal. Se identificó un alto nivel de diferenciación poblacional entre las accesiones, lo que indica que las 10 accesiones estudiadas forman poblaciones genéticamente estructuradas y que todas son muestras importantes de *M. chaseae*, que por tanto deben ser conservadas en el Banco de Germoplasma de Embrapa Pantanal. Se recomiendan cruzamientos entre las muestras con las distancias genéticas más altas para implementar planes de mejoramiento con miras a ampliar la base genética de la especie.

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Introduction

The Pantanal region, located in Mato Grosso do Sul State (MS), Central Brazilian Plateau, is the largest wetland in the world and one of the most diverse nature reserves on the planet; it is considered as a World Biosphere Reserve and Natural Heritage by UNESCO. This region is characterized by large pasture fields, with 95% of its area occupied by extensive cattle farms (Godoi Filho 1986). While many exotic species have been introduced to increase productivity, little effort has been made to understand and estimate the potential use of native species such as the grass *Mesosetum chaseae* Luces (Poaceae family). The most widely sown exotic forage has been the African grass *Urochloa humidicola* (syn. *Brachiaria humidicola*) due to its tolerance of poor soils, flooding and trampling, resulting in increases in carrying capacity of the land and farm profitability. However, monocultures in plant communities often result because of the exclusive use of this species, combined with its ability to spread in different landscapes of the Pantanal (Santos et al. 2011). Although scientific research has shown that the effect of *U. humidicola* on plant diversity is influenced by season and topography in areas experiencing seasonal flooding (Bao et al. 2015), the situation can be different in flood-free areas with soil fertility problems, mainly savannas (Pivello et al. 1999), because few plant species are adapted to such conditions, mainly native grasses (Santos et al. 2011).

Mesosetum chaseae is an important native forage plant in the Pantanal region, and is found mainly in flood-free and temporarily flooded grasslands, showing habitat plasticity and vegetative propagation in these environments (Santos et al. 2002; Alvarez et al. 2004). It is known as "grama-do-cerrado" (grass of the savanna) and is one of the most prominent forage species owing to its productivity (Santos 2001), acceptability by animals (Santos et al. 2002) and drought resistance (Santos et al. 2005). The "grama-do-cerrado" displays good tillering and grows well on poor soils. As it is prevalent under extreme climatic conditions (e.g. severe drought), this species has been considered to have potential for reclaiming degraded areas in the Pantanal. Annual aboveground dry matter production of 2,000-3,800 kg/ha has been recorded under good conditions (Santos et al. 2011), and Pinheiro et al. (2005) showed that *M. chaseae* retained forage quality during ripening and could be used for hay-making.

Studies on quality of seeds, cytogenetics and reproduction (Silva et al. 2011; 2012; 2013) represent the few investments that have been recently made for the characterization of *M. chaseae*. In the literature we found no records of molecular level studies. Investigations of genetic divergence at the molecular level of this species, however, are important to guide proposals to: 1) identify native forages for further testing to measure their suitability for sowing with or as an alternative to exotic forages, mainly *Urochloa humidicola*; and 2) implement plans for improvement of *M. chaseae* with samples maintained in the Germplasm Bank (Banco Activo de Germoplasma, BAG) of Embrapa Pantanal (Empresa Brasileira de Pesquisa Agropecuária in Corumbá, MS, Brazil). The current study aimed to make a start to document the genetic diversity of the species, and check how the accessions of *M. chaseae* from the BAG of Embrapa Pantanal are genetically structured to guide further testing in the field and future breeding programs. If suitable accessions can be identified it could provide a platform for promoting the conservation of biodiversity in pastures and the sustainability of native forage resources. Genetic polymorphism in loci for α- and β-esterase isozymes was analyzed to estimate genetic diversity and structure of the *M. chaseae* accessions.

Material and Methods

Samples of *M. chaseae* were collected from different locations in Nhecolândia, a sub-region of the Brazilian Pantanal, and are maintained in the germplasm collection at Embrapa Pantanal (Figure 1). Table 1 indicates the original collection sites according to the Universal Transverse Mercator (UTM) system.

Seeds from each *M. chaseae* accession were collected and distributed for germination in 500-mL pots containing sterile soil. The resulting plants were kept at room temperature, watered daily and used for the study. Leaves were collected from 10–30 plants of each accession; the plants were individually evaluated through electrophoresis. Leaf pieces (200 mg) from each plant were homogenized with a glass rod in an Eppendorf micro-centrifuge tube with the use of 60 µL extraction solution prepared with 1.0 M phosphate buffer pH 7.0, containing 5% PVP-40, 1.0 mM EDTA, 0.5% β-mercaptoethanol and 10% glycerol solution and maintained in a cold chamber. After homogenization, the samples were centrifuged at 25,000 rpm (48,200 x g) for 30 min, at 4 °C, in a Juan 23 MR*i* (Thermo Scientific, USA) centrifuge, and the supernatant was used for each sample.

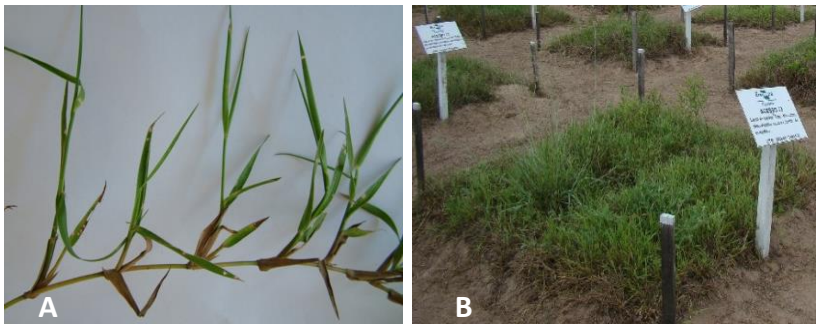

Figure 1. Tillers of *Mesosetum chaseae* (A) and accessions (B) maintained in the germplasm collection of Embrapa Pantanal, Nhumirim Farm, Nhecolândia sub-region of the Pantanal (Corumbá, MS, Brazil).

Table 1. Accessions of *Mesosetum chaseae* maintained in the germplasm collection of Embrapa Pantanal, location of the collection, habitat and geographic coordinates.

Accession	Location of the collection	Habitat	Geographic coordinates
A1	Nhumirim Farm wintering area 2	Non-flooded open grassland	19°00'04"S 56°37'09"W
A4	Campo Dora Farm wintering area 10	Seasonally flooded open grassland	18°58'55"S 56°40'55"W
A5	Chatelodo Farm wintering area 10	High open grassland	19°04'37"S 56°40'23"W
A8	Valdir's Field wintering area 10	Non-flooded savanna	18°56'12"S 56°36'56"W
A9	Valdir's Field wintering area 10	Edge of woodland	18°56'09"S 56°36'57"W
A11	Nhumirim Farm Reservation	Non-flooded open grassland	18°58'57"S 56°40'55"W
A17	Nhumirim Farm Reservation	Savanna grassland	19°04'26"S 56°40'19"W
A24	Nhumirim Farm Reservation	Non-flooded open grassland	18°58'03"S 56°37'36"W
A31	Nhumirim Farm Reservation	Savanna grassland	18°58'24"S 56°33'21"W
A32	Nhumirim Farm Reservation	Non-flooded open grassland	19°00'12"S 56°36'24"W

Polyacrylamide gels (12%) were used to analyze the esterase isozymes (EST; EC 3.1.1) according to the method of Frigo et al. (2009). The polyacrylamide gel was prepared with 0.375 M Tris-HCl, pH 8.8 as buffer. A 6.2-mL volume of acrylamide/bis-acrylamide solution (30 g acrylamide and 0.8 g bis-acrylamide dissolved in 100 mL of double-distilled water), 4.0 mL 1.5 M Tris-HCl, pH 8.0, 6.2 mL double-distilled water, 320 µL ammonium persulfate 2% and 16 µL TEMED [Tetramethylethylenediamine] were used to separate the gel. The stacking gel was prepared with 3.0 mL acrylamide/bis-acrylamide (5 g acrylamide and 0.25 g bis-acrylamide dissolved in 50 mL double-distilled water), 3.0 mL 0.24 M Tris-HCl, pH 6.8, 30 mL double-distilled water, 250 µL ammonium persulfate (2%) and 3 µL TEMED.

The electrophoresis (PAGE, polyacrylamide gel electrophoresis) was performed for 5 h at 4 °C, and a constant voltage of 200 V. The running buffer was 0.125 M Tris/0.0959 M glycine, pH 8.3.

The esterases were identified through procedures previously described by Sala et al. (2011). The gels were soaked for 30 min in 50 mL 0.1 M sodium phosphate, pH 6.2, at room temperature. Esterase activity was visualized by placing the gels for 1 h in a staining solution prepared with 50 mL of sodium phosphate solution, 30 mg of β-naphthyl acetate, 40 mg of α-naphthyl acetate, 60 mg of Fast Blue RR salt and 5 mL of N-propanol. The polyacrylamide gels were dried as described by Sala et al. (2011), and kept at room temperature for 60 min in a mixture of 7.5% acetic acid and 10% glycerol embedded in 5% gelatin. They were then placed between 2 sheets of wet cellophane paper stretched on an embroidering hoop and left to dry for 24–48 h.

The genetic variability in the *M. chaseae* samples was analyzed with POPGENE 1.32 Computer Software (Yeh et al. 1999) for the analysis of allele frequencies, mean observed heterozygosity (H_o) and expected heterozygosity (H_e) and mean number of alleles per locus (N_a), effective number of alleles per polymorphic locus (N_e), percentage of polymorphic loci (%P), χ^2 test for deviation from Hardy-Weinberg equilibrium. The *F* statistic of Sewall Wright (Wright 1965), the deficit of heterozygotes (F_{IS} and F_{IT}), and the genetic diversity between the 10 samples (F_{ST}) were also estimated using POPGENE 1.32. To explore the hierarchical partitioning of genetic variation within and among the samples, we performed an Analysis of Molecular Variance

(AMOVA, GenAlEx 6.2; Peakall and Smouse 2006). The genetic identity (Nei 1973) and distances among the *M. chaseae* samples were calculated by UPGMA grouping. Polymorphisms from α/β-esterase loci were also analyzed using STRUCTURE software 2.0 (Pritchard and Wen 2003), which evaluates the level of genetic admixture between the 160 *M. chaseae* plants. The genotypes were clustered, with the number of clusters (K) ranging from 2 to 10, and were tested using the admixture model with a burn-in period of 5,000 repeats followed by 50,000 Markov Chain Monte Carlo (MCMC) repeats, considering the presence and absence of alleles across the sample. The true number of populations (K) is often identified using the maximal value of Δ (K) returned by the software. The most probable number (K) of sub-populations was identified as described by Evanno et al. (2005). The display of the graphical output of the results STRUCTURE was taken as input data using the STRUCTURE HARVESTER, a website and software that are used to visualize STRUCTURE outputs and to implement the Evanno method (Earl and von Holdt 2012) to display a graphical representation.

Results

Native PAGE analysis for esterase isozymes in leaves of *M. chaseae* recorded with α-naphthyl acetate and β-naphthylacetate displayed α/β-esterases produced from 4 clearly defined loci and isozymes with undefined polymorphism on the fifth locus (Figure 2). The α/β-esterases were numbered in sequence, starting from the anode, according to their decreasing negative charge. The number of loci and alleles were not identified in the case of esterase isozymes with lesser anodic migration. Three alleles were detected at the *Est-1* locus, 5 alleles at the *Est-2* and *Est-3* loci, and 4 alleles at the *Est-4* locus from the leaves of the *M. chaseae* samples; allele variations were not clarified for the *Est-5* locus. Allele frequencies were analyzed for *Est-1*, *Est-2*, *Est-3* and *Est-4* loci; the estimated proportion of polymorphic loci in the *M. chaseae* accession is 100%.

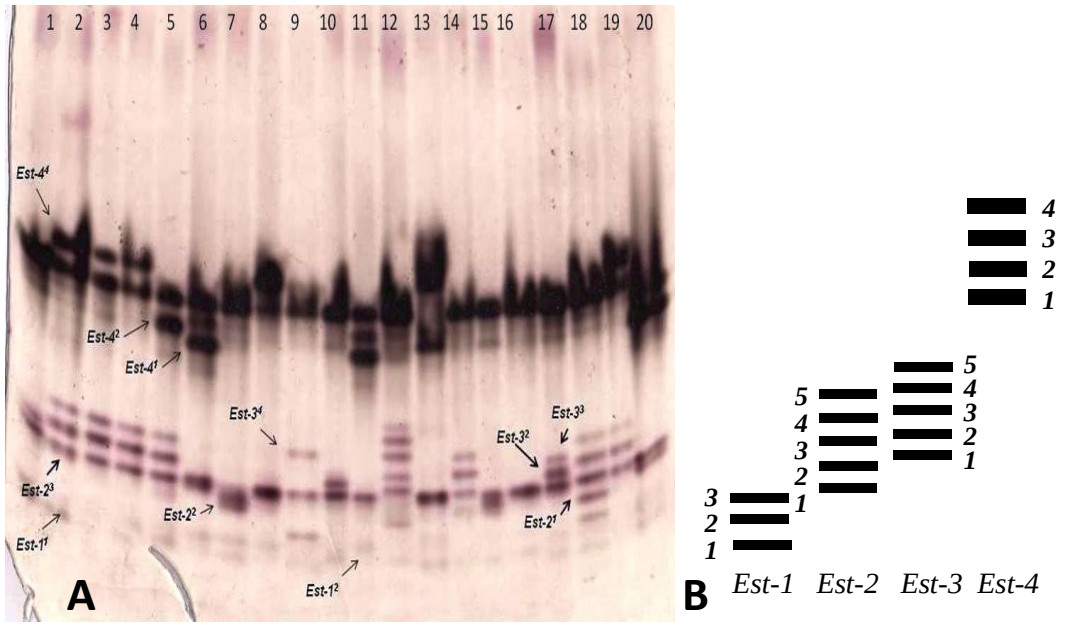

Figure 2. Polymorphism of α- and β-esterases detected in A4 (samples 1–10) and A8 (samples 11–20) accessions of *Mesosetum chaseae* produced from 4 esterase loci. Gel **A** with A4 samples: **1)** *Est-1*$^{1/2}$, *Est-2*$^{3/3}$, *Est-3*$^{1/2}$, *Est-4*$^{3/3}$; **2)** *Est-1*$^{1/1}$, *Est-2*$^{3/5}$, *Est-3*$^{4/4}$, *Est-4*$^{3/4}$; **3)** *Est-1*$^{1/2}$, *Est-2*$^{3/5}$, *Est-3*$^{4/4}$, *Est-4*$^{3/4}$; **4)** *Est-1*$^{1/2}$, *Est-2*$^{3/5}$, *Est-3*$^{4/4}$, *Est-4*$^{3/4}$; **5)** *Est-1*$^{2/2}$, *Est-2*$^{3/5}$, *Est-3*$^{4/4}$, *Est-4*$^{2/3}$; **6)** *Est-1*$^{1/2}$, *Est-2*$^{3/3}$, *Est-3*$^{4/4}$, *Est-4*$^{1/3}$; **7)** *Est-1*$^{1/2}$, *Est-2*$^{2/2}$, *Est-3*$^{1/1}$, *Est-4*$^{3/3}$; **8)** *Est-1*$^{2/2}$, *Est-2*$^{3/3}$, *Est-3*$^{1/1}$, *Est-4*$^{3/4}$; **9)** *Est-1*$^{1/3}$, *Est-2*$^{1/3}$, *Est-3*$^{4/4}$, *Est-4*$^{3/3}$; **10)** *Est-1*$^{1/1}$, *Est-2*$^{3/3}$, *Est-3*$^{2/2}$, *Est-4*$^{3/3}$. A8 samples: **11)** *Est-1*$^{2/2}$, *Est-2*$^{3/3}$, *Est-3*$^{1/1}$, *Est-4*$^{1/3}$; 1**2)** *Est-1*$^{1/1}$, *Est-2*$^{4/5}$, *Est-3*$^{4/5}$, *Est-4*$^{3/3}$; 1**3)** *Est-1*$^{1/2}$, *Est-2*$^{3/3}$, *Est-3*$^{1/1}$, *Est-4*$^{2/4}$; 1**4)** *Est-1*$^{1/1}$, *Est-2*$^{4/4}$, *Est-3*$^{3/4}$, *Est-4*$^{3/3}$; 1**5)** *Est-1*$^{1/1}$, *Est-2*$^{2/2}$, *Est-3*$^{1/1}$, *Est-4*$^{3/3}$; **16)** *Est-1*$^{1/2}$, *Est-2*$^{3/3}$, *Est-3*$^{1/1}$, *Est-4*$^{3/3}$; 1**7)** *Est-1*$^{1/2}$, *Est-2*$^{3/3}$, *Est-3*$^{2/3}$, *Est-4*$^{3/3}$; 1**8)** *Est-1*$^{1/3}$, *Est-2*$^{1/3}$, *Est-3*$^{3/4}$, *Est-4*$^{3/3}$; 1**9)** *Est-1*$^{1/2}$, *Est-2*$^{3/5}$, *Est-3*$^{4/4}$, *Est-4*$^{3/4}$; 2**0)** *Est-1*$^{1/3}$, *Est-2*$^{2/2}$, *Est-3*$^{1/1}$, *Est-4*$^{2/3}$. In **B**: the possible alleles in each *Est-1* (3), *Est-2* (5), *Est-3* (5) and *Est-4* (4) locus for α- and β-esterases detected in the 10 accessions of *M. chaseae*.

A comparison between the diversity parameters in the 10 accessions (Table 2) showed that the number of effective alleles is higher in A11 (N_e = 2.7383) than in the other *M. chaseae* accessions. The observed and expected mean heterozygosities were highest in accession A32 (H_o = 0.75; H_e = 0.5972). Departure from Hardy-Weinberg equilibrium was observed in 28 of 40 tests (70%) in 10 accessions, resulting from an excess of heterozygous samples at the *Est-1*, *Est-3* and *Est-4* loci (Table 3); the fixation index (F_{IS}) was negative at the *Est-1*, *Est-3* and *Est-4* loci. An excess of heterozygous plants (H_o>H_e) occurred in accessions A11, A17, A31 and A32 (Table 2). The deficit of heterozygous plants due to inbreeding within sub-populations was only 4.3% (F_{IS} = 0.0432) (Table 3).

A relatively high level of population differentiation was detected among the *M. chaseae* accessions (F_{ST} = 0.1693), which suggests that the 10 accessions form genetically structured populations (Table 3). The gene flow estimate calculated from F_{ST} (Nm) was also high (Nm = 1.2270) for the alleles from *Est-1*, *Est-2*, *Est-3* and *Est-4* loci. The UPGMA dendrogram obtained from the cluster analysis of Nei's (1978) unbiased genetic distance (Figure 3) reveals the formation of 2 main groups: 1 larger group was formed by 2 sub-groups comprising: i) accessions A1, A4, A5, A8 and A9, and ii) accessions A11 and A17; and the second group comprising accessions A31 and A32. Accession A24 formed an isolated group. Nei's identity (I) values ranged from 0.4839 (between accessions A9 and A11) to 0.9837 (between accessions A4 and A5) (Table 4). AMOVA showed higher genetic variation within (73%; Sum of Squares = 334,556; Variance Components = 2.23) than among (27%; Sum of Squares = 138,475; Variance Components = 0.834) the *M. chaseae* accessions.

The correlation between the genetic distance matrix and the cophenetic matrix calculated by the Mantel test (Rohlf 1989) was low (r^2 = 0.004 for 1,000 permutations), indicating that the level of genetic differentiation between frequencies of alleles in the *Est-1*, *Est-2*, *Est-3* and *Est-4* loci of the plants from the 10 *M. chaseae* accessions was not related to the geographical distance separating the plants.

The clustering of the 160 plants from 10 accessions according to a model-based Bayesian algorithm is shown in Figure 4. Each bar in the graph represents a plant and its inferred proportion of allele admixture. The colors represent 3 different clusters corresponding to differential allele proportions in each plant.

Table 2. Number of alleles (N_a) and number of effective alleles (N_e) per polymorphic locus, mean observed heterozygosity (H_o) and expected heterozygosity (H_e), and % of polymorphic α/β-esterase loci in the plants of 10 accessions of *Mesosetum chaseae* maintained in the germplasm collection of Embrapa Pantanal.

Accession	N	N_a	N_e	H_o	H_e	P%
A1	10	3.00	2.3837	0.5000	0.5188	100
A4	10	3.25	2.1450	0.4500	0.5150	100
A5	10	3.75	2.1900	0.4667	0.5004	100
A8	10	2.00	2.0983	0.4000	0.5075	100
A9	26	3.25	2.1605	0.3077	0.3937	75
A11	20	3.50	2.7383	0.5750	0.5394	100
A17	20	3.25	2.1799	0.5500	0.5151	100
A24	14	3.00	2.4541	0.3750	0.5364	100
A31	20	3.50	2.1843	0.5125	0.5000	100
A32	20	3.25	2.5453	0.7500	0.5972	100

Table 3. Number of alleles (N_a), number of effective alleles (N_e), mean observed heterozygosity (H_o) and expected heterozygosity (H_e) at *Est-1*, *Est-2*, *Est-3* and *Est-4* loci, fixation coefficients *F* (F_{IS}, F_{IT}, F_{ST}; Wright 1965) and gene flow (Nm), in plants of 10 accessions of *Mesosetum chaseae*.

Locus	N	N_a	N_e	H_o	H_e	F_{IS}	F_{IT}	F_{ST}	Nm*
Est-1	160	3.00	2.3311	0.3937	0.5753	-0.0409	0.2549	0.2842	0.6297
Est-2	159	5.00	4.2614	0.3648	0.7653	0.4744	0.5429	0.1314	1.6675
Est-3	159	5.00	2.9681	0.7484	0.6631	-0.2155	-0.0798	0.1117	1.9885
Est-4	160	4.00	1.8492	0.4750	0.4592	-0.2164	-0.0048	0.1740	1.1872
Mean	160	4.25	2.8525	0.4955	0.6147	0.0432	0.2052	0.1693	1.2270

*Nm = Gene flow [Fst = 1/(4Nm+1)]

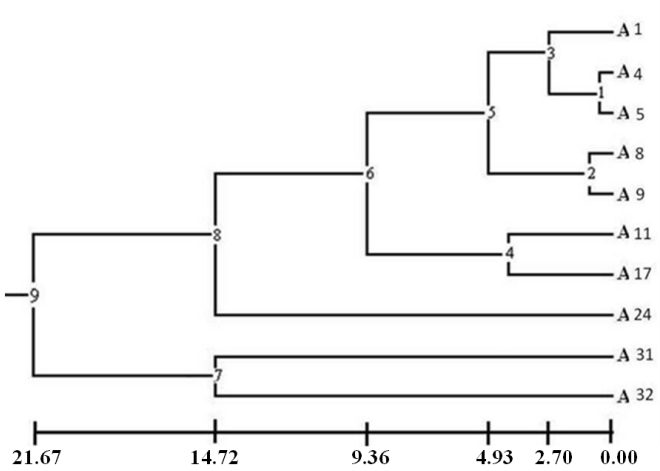

Figure 3. Similarity among the 10 accessions of *Mesosetum chaseae* from the Nhecolândia sub-region maintained in the germplasm collection of Embrapa Pantanal, based on UPGMA cluster analysis (Nei 1978) of the allele polymorphism at *Est-1*, *Est-2*, *Est-3* and *Est-4* loci.

Table 4. Dendrogram-based Nei's (1978) genetic distance: Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

	A1	A4	A5	A8	A9	A11	A17	A24	A31	A32
A1	---	0.9391	0.9561	0.6756	0.8837	0.6069	0.8927	0.8602	0.9155	0.8393
A4	0.0628	---	0.9837	0.7030	0.9333	0.6234	0.9053	0.8489	0.9085	0.7195
A5	0.0449	0.0165	---	0.7931	0.8590	0.7105	0.8929	0.8143	0.9212	0.7885
A8	0.3922	0.3524	0.2318	---	0.5313	0.7450	0.7126	0.6096	0.6905	0.5621
A9	0.1236	0.0690	0.1520	0.6324	---	0.4839	0.8135	0.9207	0.8456	0.6898
A11	0.4993	0.4726	0.3418	0.2944	0.7260	---	0.8063	0.5026	0.8118	0.6542
A17	0.1135	0.0995	0.1133	0.3388	0.2064	0.2153	---	0.7174	0.9774	0.6611
A24	0.1506	0.1638	0.2055	0.4950	0.0827	0.6879	0.3321	---	0.7394	0.7660
A31	0.0883	0.0960	0.0820	0.3704	0.1677	0.2085	0.0229	0.3019	---	0.7442
A32	0.1752	0.3293	0.2376	0.5760	0.3714	0.4243	0.4139	0.2665	0.2954	---

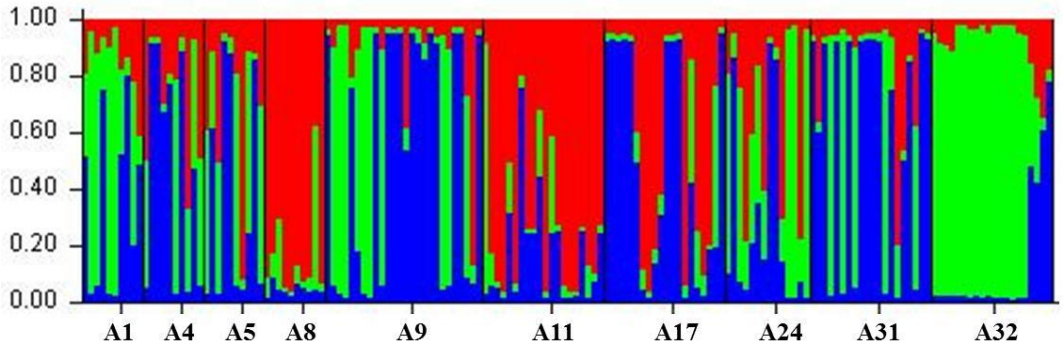

Figure 4. Bar plot-like population structure, based on polymorphism at the *Est-1*, *Est-2*, *Est-3* and *Est-4* loci for 10 accessions (A1, A4, A5, A8, A11, A17, A24, A31 and A32) of *Mesosetum chaseae* from the Nhecolândia sub-region maintained in the germplasm collection of Embrapa Pantanal within the K clusters. Each plant is represented by a single vertical bar broken in K colored segments (K = 3), with lengths proportional to each of the K inferred clusters. Each color represents the proportion of fragments for each individual, represented by a vertical bar.

Table 5. Proportion of the plants from 10 accessions of *Mesosetum chaseae* from the Nhecolândia sub-region maintained in the germplasm collection of Embrapa Pantanal in each group (K = 3), and number of plants per accession.

Accession	Group			Number of plants
	Red	Green	Blue	
A1	0.147	0.509	0.344	10
A4	0.261	0.255	0.484	10
A5	0.276	0.347	0.377	10
A8	0.835	0.120	0.046	10
A9	0.104	0.350	0.546	26
A11	0.720	0.110	0.170	20
A17	0.393	0.092	0.515	20
A24	0.296	0.492	0.275	14
A31	0.143	0.246	0.611	20
A32	0.081	0.788	0.132	20

The optimal K value determined by Bayesian analysis indicated that the plants were grouped into 3 clusters (ΔK2 = 0.00; ΔK3 = 33.7349; ΔK4 = 0.72333; ΔK5 = 1.7489; ΔK6 = 1.1321; ΔK7 = 1.5338; ΔK8 = 10.4170; ΔK9 = 0.1642; ΔK10 = 0.00). The bar plot obtained for the K value (K = 3; ΔK = 33.7349), and the results were consistent with the evidence of plants with a mixture of ancestral genome of either 2 or 3 groups, and plants with genome predominantly of 1 of the groups. At least 78% of the plants from accession A32 were in the green cluster and at least 72% of the plants of accessions A8 and A11 were in the red cluster. Plants from accessions A1, A9 and A31 contain predominantly a mixture of genome of the ancestral groups blue and green, those in A17 a mixture of ancestral groups blue and red and those in A4, A5 and A24 a mixture of genome of the groups blue, green and red (Table 5).

Bayesian analysis indicated that the most probable number of sub-populations is 3 in the *M. chaseae* accessions from the Nhecolândia sub-region kept in the germplasm collection at Embrapa Pantanal. In accession A32 there are predominantly plants ascending from one sub-population (green cluster), in accessions A8 and A11 there are predominantly plants ascending from another sub-population (red cluster), while in accessions A4, A5 and A24 there are plants ascending from the 3 sub-populations.

Discussion

The expected heterozygosity in *Est-1*, *Est-2*, *Est-3* and *Est-4* loci of *M. chaseae* ranged between 0.3937 and 0.6013 (plants from accessions A9 and A32). The mean H_e value (0.6153) of the plants from 10 accessions distributed across the Nhecolândia sub-region was high. Accession A11, showing the highest effective number of alleles (N_e = 2.7383), and A32 the highest value of mean expected and observed heterozygosity (H_e = 0.5972; H_o = 0.7500) present the highest genetic diversity. Accessions A11 and A32 may be, therefore, recommended for crossings to enlarge the genetic basis of *M. chaseae* in future breeding programs. In addition, these accessions should be planted in a range of environments to determine their suitability for increasing pasture areas planted to native species, as well as for recovering poor and degraded areas in the Pantanal region. Allendorf and Luikart (2007) suggested that the level of heterozygosity is an important indicator of the amount of adaptive genetic variation in a plant population. High heterozygosity implies a high chance for divergent selection. Populations with high heterozygosity may have been derived through different selective pressures throughout their geographical distribution and may persist under climatic or seasonal changes that may eliminate other populations. Studies of phenotypic characteristics in some samples of *M. chaseae* showed that accession A11 stands out for the large number of green leaves and a larger number of emerging leaves (Santos et al. 2007). High heterozygosity and production of green leaves are important features supporting the potential use of A11 for expansion of pastures and restoration of degraded areas, provided that it also has other desirable attributes such as high nutritional value, and adaptation to drought, mismanagement, disease and insect pests, which are yet to be evaluated.

High genetic diversity (H_e>0.50) was also detected in accessions A1, A4, A5, A8, A17, A24 and A31, indicating that these samples may have potential for use in breeding programs and warrant further study. High heterozygosity would indicate that the plant is likely to have a substantial amount of genetic variation to withstand different selective pressures. An excess of hetero-

zygous plants ($H_o>H_e$) was observed in accessions A11, A17, A31 and A32. The deficit of heterozygotes ($H_o<H_e$) was higher in A24 than in A1, A4, A5, A8 and A9. Higher density and productivity, excellent coverage with a higher number of tillers/m^2, and fewer dry leaves have been described as phenotypic characteristics for accessions A5, A9 and A24 (Santos et al. 2007).

Analyses of the genetic divergence among all 10 accessions showed a global value (F_{ST}) of 0.1693. According to Wright (1978), values of F_{ST} ranging from 0.15 to 0.25 indicate high genetic differentiation between populations. On the other hand, the estimated mean level of genetic divergence among populations for Poaceae is higher than 0.20 (Hamrick and Godt 1996). Thus, in the case of *M. chaseae*, an outcrossing grass (Silva et al. 2013), the genetic differentiation among accessions may be considered as moderate

The genetic structure observed in the accessions of *M. chaseae* is also evident in the bar plot clustering of the 160 plants from the 10 accessions showing 3 subpopulations. The 3 probable sub-populations indicated by Bayesian analysis seem not associated with the original habitat of each accession. Plants of the green group (accession A32) originated from areas of non-flooded grassland and open grassland areas, while plants of the blue group originated from savanna (accessions A17 and A31) and the edge of the cordillera (accession A9). The genetically structured populations showing genetic diversity are all important samples of *M. chaseae*, which should be maintained in the Germplasm Bank of Embrapa Pantanal.

Despite the genetic structure observed in the accessions of *M. chaseae*, the analysis of polymorphic α/β-esterase loci has indicated a significant gene flow (Nm = 1.2265) between the plants of the 10 accessions, suggesting the occurrence of outcrossing between the samples (Govindaraju 1989). The Bayesian analysis showed a mixture of plants of 3 (green, blue and red) clusters in the 10 accessions. The reproductive system in *M. chaseae* may be the main vector for the suggestive exchange of alleles. In *M. chaseae*, reproduction (tested in protected flowers) indicated allogamy (Silva et al. 2013). In addition, the high gene flow may be explained by a mixture of samples among accessions due to the dispersion of seeds that may occur in certain areas exposed to occasional flooding periods.

The genetically structured accessions of *M. chaseae* showing high genetic diversity are according to their wide distribution in the Pantanal region, their habitat plasticity and vegetative development in these environments as shown by Santos et al. (2002) and Alvarez et al. (2004). *Mesosetum chaseae* specimens had a remarkably high number of alleles per locus and greater genetic diversity than those reported within populations of other grasses (Godt and Hamrick 1998). The combinations of characteristics such as: tropical region, regional range, perennial life form, sexual mode of reproduction, outcrossing breeding system, and seed dispersal mechanism, may explain the great genetic diversity in samples of *M. chaseae.* The low number of loci analyzed and the high genetic diversity generally ascribed to the esterase isozymes may also explain the high genetic diversity observed in our study compared with other grasses (Godt and Hamrick 1998).

In *Eragrostis curvula*, a valuable native forage grass in Africa, the esterase pattern proved useful as an additional criterion for identifying the individual taxa making up the so-called *Eragrostis curvula* complex (*E. curvula, E. conferta, E. robusta, E. chloromelas* and *E. lehmanniana*) and for evaluating their reciprocal relationships (Poverene and Curvetto 1989). Isozymes (including esterases) were used for genetic characterization of different samples of elephant grass (*Pennisetum purpureum*), a species of forage widely distributed in Brazil after its introduction to the country (Daher et al. 1997), although the values for heterozygosity were not presented. When the isozymes α- and β-esterase were used to analyze the forages *Brachiaria brizantha* (now: *Urochloa brizantha*), *B. plantaginea, B. humidicola* and *B decumbens* (now: *U. plantaginea*, *U. humidicola* and *U. decumbens*, respectively), a clear distinction between the 4 species was possible (Martins et al. 1999). Using the esterase system also made it possible to identify quickly and accurately the patterns of 7 cultivars of elephant grass (*Pennisetum purpureum*) and their hybrids with Pearl millet (*P. americanum*) from the germplasm bank of elephant grass (Freitas et al. 2000).

Higher genetic variability within than between samples also has been described for other forage species of the Poaceae family such as *Bromus auleticus*, using isozymes (esterases and peroxidases) and random amplified polymorphism of DNA (Yanaka et al. 2005). Since the year 2000, besides isozymes, DNA markers have also been used as tools to investigate the genetic diversity in forages. *Stylosanthes macrocephala*, a tropical forage legume native to Brazil, showed high polymorphism of random amplified DNA segments (RAPD), but the levels of heterozygosity and genetic divergence among accessions (F_{ST}) were not reported (Barros et al. 2005). Recently, the characterization of microsatellite markers in the grass, *Paspalum atratum*, has been considered a potential and promising tool for genetic diversity studies

of *Paspalum* species (Cidade et al. 2010). Association of Single-Nucleotide Polymorphism markers with candidate genes in mapping of forage quality traits in perennial ryegrass was reported in Gill et al. (2012).

Estimates of heterozygosity are important indicators of the adaptive potential of populations for increasing grazing areas, and also for investigations for improvement of the species. Plants showing characteristics of interest (e.g. resistance to drought, greater leaf weight, early development) and high levels of heterozygosity are promising sources of inbred lines for breeding programs. As the levels of heterozygosity of the 10 accessions of *M. chaseae* have been estimated in our study, identification of features of interest for each sample would be necessary before mounting a breeding program for the species. The values of genetic identity (Nei 1973) and distances between the accessions estimated in our study (Table 4) are important information to assist in formulating a breeding program for the species. Crosses between accessions with the highest genetic distances (A9 and A11: 0.7260; A8 and A9: 0.6324; and A8 and A32: 0.5760) would broaden the genetic base of the species. Only 10 combinations among the accessions (A1 and A4, A1 and A5, A1 and A31, A4 and A5, A4 and A9, A4 and A17, A4 and A31, A5 and A31, A9 and A24 and A17 and A31) showed a very high level of genetic identity (I>0.90). Lower levels of genetic identity have been reported for other forage species: 0.0 to 0.857 and 0.2916 to 0.7142 in samples of *Pennisetum purpureum* (Daher et al. 1997; Freitas et al. 2000), and 0.0 to 0.50 in 16 samples of *Bromus auleticus* (Yanaka et al. 2005).

Analysis of the genetic polymorphism in loci for α- and β-esterase isozymes of *M. chaseae* accessions kept in the Germplasm Bank of Embrapa Pantanal was important for selecting accessions for further testing to determine their potential for use in recovering degraded areas, and for sowing as grazing pasture areas in the Pantanal region. The study has shown that, although the accessions presented a very high level of genetic identity (I>0.90), the samples form genetically structured populations (0.15<Fst<0.25). Field testing of accessions in a range of environments is warranted to determine if they possess other desirable attributes, e.g. persistence, drought-tolerance, high nutritive value, and disease- and pest-resistance, before they could be recommended for widespread sowing. Since there were no identical samples, all should be maintained in the Germplasm Bank of Embrapa Pantanal. Crossing plants from accessions with the highest genetic distances is recommended for the improvement of this species, with the prospect of broadening the genetic base and opening possibilities for the emergence of new characteristics of interest.

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References

Allendorf FW; Luikart G. 2007. Conservation and the genetics of populations. Blackwell Publishing, Malden, MA, USA.

Alvarez JM; Rocha JF; Santos SA; Machado SR. 2004. Anatomia foliar de *Mesosetum chaseae* Luces (Poaceae) vegetando em diferentes ambientes com e sem influência de pastejo na subregião da Nhecolândia, Pantanal Sul-Mato-Grossense. In: Anais, IV Simpósio sobre Recursos Naturais e Socio-Econômicos do Pantanal, 23−26 November 2004, Corumbá, MS, Brazil (CD-ROM).

Barros AM; Faleiro FG; Karia CT; Shiratsuchi LS; Andrade RP de; Lopes GKB. 2005. Varia-bilidade genética e ecológica de *Stylosanthes macro-cephala* determinadas por RAPD e SIG. Pesquisa Agro-pecuária Brasileira 40:899–909. DOI: 10.1590/s0100-204x2005000900010

Cidade FW; Souza-Chies TT; Souza FHD; Batista LAR; Dall'Agnol M; Valls JFM; Zucchi MI; Souza AP. 2010. Microsatellite loci for *Paspalum atratum* (Poaceae) and cross-amplification in other species. American Journal of Botany 97:e107–e110. DOI: 10.3732/ajb.1000207

Daher RF; Moraes CF; Cruz CD; Pereira AV; Xavier DF. 1997. Diversidade morfológica e isoenzimática em capim-elefante (*Pennisetum purpureum* Schum.). Revista Brasileira de Zootecnia 26:255–264.

Earl DA; vonHoldt BM. 2012. STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4:359–361. DOI: 10.1007/s12686-011-9548-7

Evanno G; Regnaut S; Goudet J. 2005. Detecting the number of clusters of individuals using the software Structure: A simulation study. Molecular Ecology 14:2611–2620. DOI: 10.1111/j.1365-294x.2005.02553.x

Freitas NSA; Falcão TMMA; Burity HÁ; Tabosa JN; Silva MV. 2000. Caracterização e diversidade genética do capim-elefante e seus híbridos com milheto mediante padrões isoenzimáticos. Pesquisa Agropecuária Brasileira 35:1125–1133. DOI: 10.1590/s0100-204x2000000600008

Frigo MJ; Mangolin CA; Oliveira Junior RS; Machado MFPS. 2009. Esterase polymorphism for analysis of genetic diversity and structure of wild poinsettia (*Euphorbia heterophylla*) populations. Weed Science 57:54–60. DOI: 10.1614/ws-08-096.1

Gill G; Faville M; Bryant C; Rouillard J-M; Hanley Z. 2012**.** SNP discovery and candidate gene-based association mapping of forage quality traits in perennial ryegrass**.** In: Bushman BS, ed. Proceedings of the 7th International Symposium on the Molecular Breeding of Forage and Turf. p. 24.

Godoi Filho JD. 1986. Aspectos geológicos do Pantanal Mato-Grossense e de sua área de influência. In: Anais, I Simpósio sobre Recursos Naturais e Sócio-Econômicos do Pantanal, Corumbá, MS, Brazil. p. 63–76.

Godt MJW; Hamrick JL. 1998. Allozyme diversity in grasses. In: Cheplick GP, ed. Population biology of grasses. Cambridge University Press, Cambridge, UK. p. 11–29. DOI: 10.1017/cbo9780511525445.003

Govindaraju DR 1989. Variation in gene flow levels among predominantly self-pollinated plants. Journal of Evolutionary Biology 2:173–181. DOI: 10.1046/j.1420-9101.1989.2030173.x

Hamrick JL; Godt MJW. 1996. Effect of life history traits on genetic diversity in plant species. Philosophical Transactions of the Royal Society B 351:1291–1298. DOI: 10.1098/rstb.1996.0112

Martins CC; Martins D; Mori ES; Souza FHD; Ramos PRR. 1999. Eletroforese de isoenzimas de plântulas na identificação de espécies de *Brachiaria***.** Planta Daninha 17:433–443. DOI: 10.1590/s0100-83581999000300010

Nei M. 1973. Analysis of gene diversity in subdivided populations. Proceedings of the National Academy of Sciences of the United States of America 70:3321–3323.

Nei M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89:583–590. DOI: 10.1073/pnas.70.12.3321

Peakall R; Smouse PE. 2006. GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6:288–295. DOI: 10.1111/j.1471-8286.2005.01155.x

Pinheiro LC; Santos SA; Comastri Filho JA. 2005. Produção de forragem de pastagem com predominância de grama-do-cerrado submetida a duas épocas de vedação, no Pantanal. In: Sociedade Brasileira de Zootecnia, ed. Anais, VII Congresso Internacional de Zootecnia, Campo Grande, MS, Brazil (CD-ROM).

Poverene MM; Curvetto NR. 1989. Esterase isozyme variation in the *Eragrostis curvula* complex (Poaceae). Plant Systematics and Evolution 166:173–181. DOI: 10.1007/bf00935947

Pritchard JK; Wen W. 2003. Documentation for STRUCTURE software: Version 2. http://pritch.bsd.uchicago.edu

Rohlf FJ. 1989. NTSYS-Pc: Numerical Taxonomy and Multivariate Analysis System. Exeter Publishing Co, New York, USA.

Sala J; Mangolin CA; Franzoni J; Machado MFPS. 2011. Esterase polymorphism and the analysis of genetic diversity and structure in cactus populations descended from *Cereus peruvianus* plants regenerated in vitro. Biochemical Genetics 49:270–282. DOI: 10.1007/s10528-010-9405-5

Santos SA. 2001. Caracterização dos recursos forrageiros nativos da subregião da Nhecolândia, Pantanal, Mato-Grosso do Sul, Brasil. Ph.D. Thesis. Universidade Estadual Paulista, Botucatu, SP, Brazil.

Santos SA; Costa C; Souza GSE. 2002. Composição botânica da dieta de bovinos criados em pastagem nativa na sub-região da Nhecolândia, Pantanal, Brasil. Revista Brasileira de Zootecnia 31:1648–1662. DOI: 10.1590/s1516-35982002000700007

Santos SA; Comastre Filho JA; Cardoso EL. 2005. Identificação de espécies forrageiras nativas tolerantes à seca na sub-região da Nhecolândia, Pantanal. In: Anais, VI Congresso Brasileiro de Zootecnia, 2005, Campo Grande, MS, Brazil (CD-ROM).

Santos SA; Silva LAC; Abreu UGP; Jank L; Ogihara LK; Parrili M; Matsuhara AS. 2007. Avaliação de descritores fenotípicos de populações da Grama-do-cerrado no pico de seca, Pantanal. In: Resumos, Simpósio Internacional sobre Melhoramento de Forrageiras, Campo Grande, 2007. Embrapa Gado de Corte, Campo Grande, MS, Brazil (CD-ROM).

Santos SA; Desbiez A; Crispim SMA; Comastri Filho JA; Abreu UGP; Rodela LG. 2011. 3. Natural and cultivated pastures and their use by cattle. In: Junk WJ; Silva CJ; Cunha CN; Wantzen KM, eds. The Pantanal: Ecology, biodiversity and sustainable management of a large neotropical seasonal wetland. Pensoft Publishers, Sofia − Moscow. p. 327–353. DOI: 10.13140/2.1.3056.7367

Silva LAC; Santos SA; Costa C; Meirelles PRL; Comastri-Filho JA; Garcia JB. 2011. Perfilhamento de plantas e qualidade de sementes de *Mesosetum chaseae* Luces na sub-região da Nhecolândia, Pantanal, MS. Veterinária e Zootecnia (UNESP) 18:610–620.

Silva LAC; Pagliarini MS; Santos SA; Silva N; Souza VF. 2012. Chromosome number, microsporogenesis, microgametogenesis, and pollen viability in the Brazilian native grass *Mesosetum chaseae* (Poaceae). Genetics and Molecular Research 11:4100–4109. DOI: 10.4238/2012.september.12.1

Silva LAC; Pagliarini MS; Santos SA; Valle CB. 2013. Stigma receptivity, mode of reproduction, and mating system in *Mesosetum chaseae* Luces (Poaceae), a native grass of the Brazilian Pantanal. Genetics and Molecular Research 12:5038–5045. DOI: 10.4238/2013.october.25.2

Wright S. 1965. The interpretation of population structure by F-statistics with special regard to systems of mating. Evolution 19:395–420. DOI: 10.2307/2406450

Wright S. 1978. Variability within and among populations. University of Chicago Press, Chicago, IL, USA.

Yanaka FY; Dall´Agnol M; Schifino-Wittmann MT; Dias PMB; Gomes KE. 2005. Variabilidade genética em

populações naturais de *Bromus auleticus* Trin. ex Nees (Poaceae) com base em isoenzimas e marcadores RAPD. Revista Brasileira de Zootecnia 34:1897–1904. DOI: 10.1590/s1516-35982005000600013

Yeh FC; Yang RC; Boyle T. 1999. POPGENE Version 1.31: Microsoft Window-based freeware for population genetic analysis. University of Alberta and Center for International Forestry Research.

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