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Cidade *et al.*

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# Genetic variation in polyploid forage grass: Assessing the molecular genetic variability in the *Paspalum* genus

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## Abstract

**Background:** *Paspalum* (Poaceae) is an important genus of the tribe Paniceae, which includes several species of economic importance for foraging, turf and ornamental purposes, and has a complex taxonomical classification. Because of the widespread interest in several species of this genus, many accessions have been conserved in germplasm banks and distributed throughout various countries around the world, mainly for the purposes of cultivar development and cytogenetic studies. Correct identification of germplasms and quantification of their variability are necessary for the proper development of conservation and breeding programs. Evaluation of microsatellite markers in different species of *Paspalum* conserved in a germplasm bank allowed assessment of the genetic differences among them and assisted in their proper botanical classification.

**Results:** Seventeen new polymorphic microsatellites were developed for *Paspalum atratum* Swallen and *Paspalum notatum* Flüggé, twelve of which were transferred to 35 *Paspalum* species and used to evaluate their variability. Variable degrees of polymorphism were observed within the species. Based on distance-based methods and a Bayesian clustering approach, the accessions were divided into three main species groups, two of which corresponded to the previously described Plicatula and Notata *Paspalum* groups. In more accurate analyses of *P. notatum* accessions, the genetic variation that was evaluated used thirty simple sequence repeat (SSR) loci and revealed seven distinct genetic groups and a correspondence of these groups to the three botanical varieties of the species (*P. notatum* var. *notatum*, *P. notatum* var. *saurae* and *P. notatum* var. *latiflorum*).

**Conclusions:** The molecular genetic approach employed in this study was able to distinguish many of the different taxa examined, except for species that belong to the Plicatula group, which has historically been recognized as a highly complex group. Our molecular genetic approach represents a valuable tool for species identification in the initial assessment of germplasm as well as for characterization, conservation and successful species hybridization.

**Keywords:** Cross-species amplification, Genetic diversity, Germplasm evaluation, Microsatellite markers, *Paspalum* botanical varieties

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## Background

The genus *Paspalum* L. is an important member of the Paniceae tribe (Poaceae) and includes between 330 and 400 species, most of which are native to the tropical and subtropical regions of the Americas [1-3]. *Paspalum* species can be found in diverse habitats, such as subtropical rainforests, savannas, marshes and dunes, but they are more frequently found in the natural grasslands of eastern Bolivia, Paraguay, central and southern Brazil, northern Argentina and Uruguay [3]. The main center of origin and diversity of the genus is considered to be located in the South American tropics and subtropics [1], primarily in central Brazil, where numerous species appear to be associated with savannas and rocky terrain. Brazil harbors the greatest number of *Paspalum* species [4,5], with approximately 220 species that exist in nearly all herbaceous plant communities within different ecosystems [6].

Because of this degree of complexity, the division of the *Paspalum* genus into subgenera, sections or informal groups has been proposed by many authors and has been extensively discussed [1-3,7-16]. Currently, four subgenera are recognized within *Paspalum*: *Paspalum* subg. *Anachyris* Chase, *P.* subg. *Ceresia* (Pers.) Rchb., *P.* subg. *Harpostachys* (Trin.) S. Denham and *P.* subg. *Paspalum* [3], the last of which consists of approximately 265 species divided into 25 informal groups [1,14]. However, such rankings have been based on morphological similarities, and the evolutionary and genetic relationships between these groups are not always clear. Several species, mainly within *Paspalum* subg. *Paspalum*, are of economic importance for foraging, turf and ornamental purposes [17] in different parts of the world. Dallisgrass (*Paspalum dilatatum* Poir., Dilatata group) and bahiagrass (*P. notatum* Flügge, Notata group) are especially important and are widely used for forage, mainly in the southern United States of America (USA) [18,19]. *P. atratum* Swallen (Plicatula group) has been the object of growing interest for use as forage in areas that are subjected to periodic flooding in Florida (USA), northeastern Argentina, Brazil, Thailand, the Philippines and Australia [18]. In addition, *Paspalum scrobiculatum* L. ('Kodo millet', Plicatula group) is cultivated in India as a cereal crop [3,20], and *Paspalum vaginatum* Sw. (Disticha group) [21] and *P. notatum* [19] are widely grown as turf grass.

Because of the widespread interest in several species of this genus, many accessions have been conserved in germplasm banks and distributed throughout various countries for cultivar development and cytogenetic studies. However, the use of some accessions is restricted due to difficulty with accurate taxonomical identification. In Brazil, despite constant evaluations of germplasm banks and taxonomic reviews [22], many species

of *Paspalum*, especially those included in the Plicatula Group, remain unidentified. This deficit presents a problem because the correct identification of germplasms and the quantification of their variability are necessary for the development of conservation and breeding programs.

The complexity of the taxonomical classification of *Paspalum* germplasms results from the complex evolutionary history of the group. Polyploidy occurs at a high frequency within the genus [23,24] and has played a crucial role in the evolution of *Paspalum*. Most species have  $x = 10$  as the basic chromosome number, with ploidy levels that range from diploid to hexadecaploid [25]. Diploid species are not rare within the genus [3,25-32], but nearly 80% of the investigated species are polyploids, among which 50% are tetraploid, and most of these tetraploids are apomictic [24,33]. Many *Paspalum* species comprise sexual diploid and apomictic polyploid cytotypes, and several have been shown to have arisen through natural hybridization [34]. However, interspecific hybridization and allopolyploidy are not always morphologically evident in *Paspalum* species; therefore, additional methods of taxonomic classification are required [35].

Many taxonomical and species characterization problems arise as a consequence of the great morphological variation present in agamic complexes. *P. notatum* is a good example of this situation, because it forms an agamic complex and presents wide morphological variation [7,36,37]. This species is a perennial rhizomatous turf and forage grass and is recognized as a major constituent of the native grasslands of the New World, being found from Central Eastern Mexico to Argentina and throughout the West Indies [1]. This grass is economically important and is widely used for forage production, mainly in the southern USA [19].

Various morphological and cytological forms are recognized within *P. notatum* [7,37]. This species includes several genotypes, which differ in both their ploidy levels and their reproductive systems. The diploid cytotype ( $2n = 2x = 20$ ) is sexual and self-incompatible [38,39], whereas the tetraploid cytotype ( $2n = 4x = 40$ ) is a self-compatible pseudogamous aposporous apomict [40]. Apomixis in tetraploid *P. notatum* can be either obligate or facultative [24]. In botanical terms, the tetraploid cytotypes are usually considered to be the typical form of *P. notatum*; as such, they form the variety *notatum*. On the other hand, the diploid cytotypes are classified as belonging to the *saurae* variety based on their distinct morphological characteristics [36]. In addition to these two widely recognized varieties, Döll [41] proposed the variety *latiflorum*, which was accepted by some taxonomists [36,42,43] until the mid-1980s. However, *P. notatum* var. *latiflorum* is not currently recognized.

Molecular markers are of great value in plant studies and have been used for multiple purposes, including

estimating the genetic diversity, determining accession relationships, elucidating evolutionary relationships, aiding in the taxonomic classification of many plants [44-49] and aiding in the identification of botanical varieties [50,51]. In addition, utilizing molecular markers that regularly identify many genetic polymorphisms at low taxonomic levels makes it possible to address the relationship between morphological and genotypic variation.

The molecular markers that are more informative for the discrimination of closely related genotypes include microsatellite sequence markers [52-54]. Microsatellites are tandem repeat sequences of 1 to 6 nucleotides that are widely distributed in the genome [55]. However, these markers are usually species specific, and in the *Paspalum* genus, few microsatellite markers are available for *P. vaginatum* [56], *P. dilatatum* and the related species [57], *P. notatum* [58] and *P. atratum* [59]. However, primers designed for source species have been successfully employed to amplify nuclear SSRs in closely related taxa when the DNA regions that flank the microsatellite loci are sufficiently conserved [57,59-67].

The main objectives of this study are (1) to evaluate the informative potential of SSRs for genetic discrimination in different species of *Paspalum* using markers developed for *P. atratum* and *P. notatum*; (2) to conduct an in-depth study of the extent, distribution and structure of the genetic variation of *P. notatum* in a South American collection of this species; and (3) to evaluate the genetic patterns and relationships among different morphological types found in this species and correlate them with their geographic distribution.

## Methods

### Plant material and DNA extraction

A total of 214 accessions of *Paspalum* (177 accessions sampled from 35 species and 37 unclassified accessions) were included in this study. These accessions have been maintained *in vivo* at Embrapa Southeast Livestock, located in São Carlos, São Paulo (SP), Brazil and at the Federal University of Rio Grande do Sul in Porto Alegre, Rio Grande do Sul (RS), Brazil (Additional file 1). Fifty-seven *P. notatum* accessions from germplasm collections were evaluated in a more detailed analysis in this study (Additional file 2). The genomic formula, chromosome number, type of polyploidy and mode of reproduction are presented for all *Paspalum* species investigated in this study (Additional file 3).

Genomic DNA from each sample was isolated from lyophilized young leaf tissue using the cetyltrimethyl ammonium bromide (CTAB) method proposed by Doyle and Doyle [68] with minor modifications. DNA concentrations were estimated by comparison with known concentrations of  $\lambda$  DNA on 0.8% agarose gels.

### Development of new SSR markers for *Paspalum* and SSR analysis

A microsatellite-enriched genomic library was obtained for *P. notatum* using the method described by Billotte et al. [69]. The steps for obtaining the clones, sequencing and analysis, the determination of the criteria for selecting the microsatellites, the primer design and the amplification were conducted according to previously described methodology [58]. For some loci, a touchdown protocol was used, as previously described [70]. Only the strongest bands were considered because of the possibility that lighter bands could be stutter bands that result from the slippage of Taq polymerase during the PCR amplifications [63,71].

Cross-species amplification was tested for 214 accessions that correspond to 35 *Paspalum* species, with a set of 23 SSR primer pairs developed for *P. atratum* and *P. notatum* [58,59] and new SSRs developed in this work. An SSR was considered to be transferable when a band of the expected size was amplified via PCR and an SSR pattern was observed. To avoid false negatives, primers that resulted in null alleles in some of the samples were tested at least twice. The microsatellite loci that were amplified successfully and that showed readable electrophoretic patterns and an absence of nonspecific products were used for the genetic evaluation of the germplasm collection.

A more detailed and accurate analysis of the *P. notatum* accessions was conducted using 15 microsatellite markers developed by Cidade et al. [58,59] and 15 new SSRs developed in this work.

The total *Paspalum* germplasm and the *P. notatum* accessions were genotyped and analyzed in the same manner. No assumptions were made about the genetic nature of the examined alleles because of the high ploidy levels of the majority of the *Paspalum* accessions. Hence, each SSR allele was treated as dominant in this study due to the high ploidy levels of the samples [72]; these alleles will hereafter be referred to as bands. Each allele was scored as 1 (present) or 0 (absent) and was arranged in a matrix. Non-amplified loci were scored as missing data. Summary statistics, including the total number of bands, the number of bands per locus, and the polymorphism information content (PIC) [72] were determined based on the data matrix that documented the microsatellite genotyping of each locus. To verify the information obtained for these SSR loci for future genetic studies of other *Paspalum* species, the number of bands and the PIC values were calculated for each marker within each species.

### Clustering and population structure analyses

For the analysis of the *Paspalum* germplasm, genetic similarity (GS) indices were calculated for all the

possible pairwise comparisons using Dice's similarity coefficient [73]. For calculation of the *P. notatum* GS indices, Jaccard's similarity index was used [74]. All of the calculations were performed with the software package NTSYS-pc 2.1 [75]. For both analyses, clustering was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) [76], and the significance of the cophenetic correlation was tested with the Mantel correspondence test [77]. Additionally, a PCO was conducted.

An unrooted dendrogram was constructed for both analyses using the weighted neighbor-joining method (NJ) with DARwin 5.0.157 software [78], and trees were drawn with Figtree v. 1.3.1 software [79]. The bootstrap method was employed to evaluate the reliability of the tree topology. Bootstrap calculations were performed using BOOD 3.0 [80] and DARwin 5.0.157 [78] software based on 1000 replications. The cophenetic coefficient between the matrix of genetic similarity and the dendrogram was computed using NTSYS-pc 2.1 software [75].

The program STRUCTURE, version 2.3.2 [81-83], was used to detect the population structure and to assign individuals to subpopulations. This program employs model-based clustering in which a Bayesian approach identifies groups based on compliance with Hardy-Weinberg equilibrium and linkage equilibrium. In this study, membership in each genotype was tested for the range of genetic groups, from  $K = 1$  to  $K = 12$ , with the admixture model and without prior information regarding their origin, using 300,000 replicates for burn-in and 500,000 replicates for Markov Chain Monte Carlo processes for each run. Analyses were conducted with correlated allele frequencies (AFC) for the putative populations. The final subgroups were determined based on the ad hoc measure  $\Delta K$  [84], which was calculated using an application on the STRUCTURE Harvester website [85].

The binary matrix derived from the SSR data was examined via an analysis of molecular variance (AMOVA) using Arlequin 3.11 software [86]. The partitioning of the genetic variation within *Paspalum* was calculated, and the significance of each variation was estimated non-parametrically using 1000 permutations. An AMOVA was also performed to evaluate the differentiation between the *P. notatum* groups obtained from STRUCTURE. Furthermore, the genetic distances among these groups were calculated as the Slatkin distance and pairwise  $\phi_{ST}$ .

#### Morphological traits of *Paspalum notatum*

Data regarding nine morphological traits from [87] and unpublished data from Batista (2008) were used for the morphological analysis of *P. notatum*. The following vegetative traits were included in this analysis: leaf sheath length (LSL), leaf sheath width (LSW), leaf blade length (LBL), leaf blade width (LBW) and leaf blade

hairiness (LBH). In addition, the following four reproductive traits were examined: inflorescence stem length (ISL), average length of inflorescence branches (ALIB), spikelet length (SL) and spikelet width (SW). The plant material was botanically identified according to Parodi [36], Barreto [7] and Canto-Dorow [37].

A data matrix was created that included 46 accessions and nine morphological traits, which comprised a mixed matrix that was composed of one qualitative and eight quantitative traits. The data were standardized by dividing each real value by the standard deviation of the trait. A PCO was performed, and descriptive statistics were calculated using GENES software [88]. Pairwise matrices of Euclidean distance were calculated using NTSYS-pc 2.1 software [75]. Based on the results obtained via principal coordinate analysis (PCA) and PCO, a graph was constructed using STATISTICA software, version 7.0 [89].

To estimate the correlation between the morphological distances and molecular similarities, a Jaccard's similarity matrix was constructed based on molecular genotyping of the morphologically evaluated accessions. Correlations were calculated using the Mantel test [77] in NTSYS-pc 2.1 software [75].

#### Geographic origin of *Paspalum notatum*

The geographic coordinates of the *P. notatum* accessions were used to construct a matrix of linear distances (in kilometers) between pairs of genotypes using Geographic Distance Matrix Generator software [90]. The correlation between this matrix and the genetic similarity matrix (generated using microsatellites) was tested with NTSYS-pc 2.1 software using the Mantel test. The geographic distribution of the accessions was visualized with the DIVA-GIS program, version 5.2.0.2 [91].

## Results

#### Development of new SSR markers for *Paspalum* and SSR analysis

Seventeen new SSR polymorphic loci were developed for the investigated *Paspalum* species (Table 1), among which 15 were isolated from *P. notatum* and two from *P. atratum*. A total of 121 bands were scored; the number of bands per locus ranged from 4 to 15, with an average of 7.12 bands per locus (Table 2).

Of the 23 microsatellite primer pairs tested for transferability to other species, 12 (52%) successfully amplified their respective loci in most species (Additional file 4). Exceptions were found for the PA01-C8 locus, which showed weak amplification in *Paspalum ovale* Nees ex Steud., and the PN03-A6 locus, which showed weak amplification in *Paspalum pumilum* Ness, *Paspalum subciliatum* Chase, and *P. ovale*. All 12 of the SSRs that transferred to other species revealed a high degree of SSR polymorphism within the surveyed species, which

**Table 1 New SSR loci developed for Paspalum**

Locus name	Repeat motif	Ta (°C)	Product size (bp)	NM	PIC	F (5'-3')	R (5'-3')	GenBank accession number
PN02-B3	(GT)12	50	139-157	11	0,8	CAAACAATGGGACAACACTT	TTTTTCTGCTCTGGCTCAT	HQ585421
PN02-C6	(TG)8	51,4	139-145	4	0,3	TTCAGCAATCAAGAGTTAGA	AGAGACGATCAGGGTGTG	HQ585422
PN02-G3	(GT)9	60	294-302	5	0,6	GTCGCCGGTGCAGTCG	CACATAGCCGGCCTCCTCTC	HQ585423
PN02-G7	(AC)7	TOUCHDOWN	118	7	0,7	TGCAGCATGTAATAACC	TACACAGGAAGGAAGAAA	HQ585424
PN03-C7	(GT)6	50	159-165	3	0,6	TCATGCCACGAGAAAGAAACCA	CAGGGACGACAGGGACAAGACT	HQ585425
PN03-D10	(GT)11	50	280-300	7	0,7	GTTATTCCTTCACTCACTACC	AGCTTTCTGCCTTCTTTTT	HQ585426
PN03-D12	(AG)10	50	220-240	7	0,7	TGGGGAAGCAGGAAGTCACA	CAGCTCACCATGGGAATG	HQ585427
PN03-E7	(GT)9	TOUCHDOWN	298	7	0,7	CATGCTTTTCTGCTTCC	GCTAATACTGCTTGCTCTC	HQ585428
PN03-F1	(CA)7	50	184-192	5	0,7	CAGTGACCAAGCTTACAACCTA	GTGGCATGCCCTTCTACAA	HQ585429
PN03-F10	(TG)7	60	165-177	7	0,8	TGCCTACCGTTTCTCTTCTCT	GGGATGGTCTTGACTCTTGAT	HQ585433
PN03-F3	(GT)8	60	165-203	8	0,8	ACCCCGCTCCTCTTTCTCA	CCCAACCACCATTATCTCTG	HQ585430
PN03-F7	(CA)10	50	200-212	6	0,8	TGCTGGCAGTCTTCTCT	GGCCATACCCTTTTTG	HQ585431
PN03-F9	(CA)8	60	179-275	15	0,9	AAAAGGGCAGGAGTTAGGAC	AGTTGGCTCAGTTACGATTTTA	HQ585432
PN03-G5	(CA)7	60	126-138	6	0,7	AAGTCACCGTTTCAATCCAG	GCAGTGCGCAAGTTTTCT	HQ585434
PN03-H3	(GA)22	51,4	198-250	11	0,8	CATGTAAACGTAGCAGAGGT	GCTAATTTTACAGGGTGGTC	HQ585435
PA01-C8	(AT)2TTA(AT) 2TTCA(GA)2	60	247-279	6	0,8	AATCTGACCTGTTTACTTCTC	TGCATTTTTGGGATACACT	HM208140
PA01-E10	(GGT)2 T(ACT)2	54	186-210	6	0,4	GCGATTATTGTTTGGTTTG	ACGCTTCTTGCTTCTCATCTCA	HM208141
Average					7,1	0,7		

Ta primer annealing temperature, TD touchdown, NM number of markers, PIC polymorphism information content. PN *Paspalum notatum* SSR loci, PA *P. atratum* SSR loci.

allowed an analysis of the organization of genetic diversity within the *Paspalum* germplasm to be conducted. The mean PIC was 0.65, and an average of 15.58 bands per locus was found (Table 2). The PA02-B1, PA01-B7, PN03-C7, PN03-A6, and PN02-B5B loci were the most informative, with more than 20 bands being amplified for each of these loci (Additional file 4).

Among the *P. notatum* accessions, the 30 microsatellite primer pairs evaluated amplified a total of 208 bands, with an average of 6.93 fragments per locus and a mean PIC of 0.64 being obtained (Table 2). The combined electrophoretic profile of all of the loci revealed 44 distinct genotypes, which suggests that there were duplicates or clones in the analyzed material.

#### Clustering analyses in the *Paspalum* genus

All of the analyses performed supported the formation of three major groups in the evaluated *Paspalum* germplasm (Figures 1, 2 and 3), which corresponded to the *Paspalum* botanical groups Notata and Plicatula and a group formed by all the other species of *Paspalum*. Because of the differences between the matrices obtained when analyzing different microsatellite loci, we will address only the major groups and the most robustly supported groups derived from distinct methods of analysis.

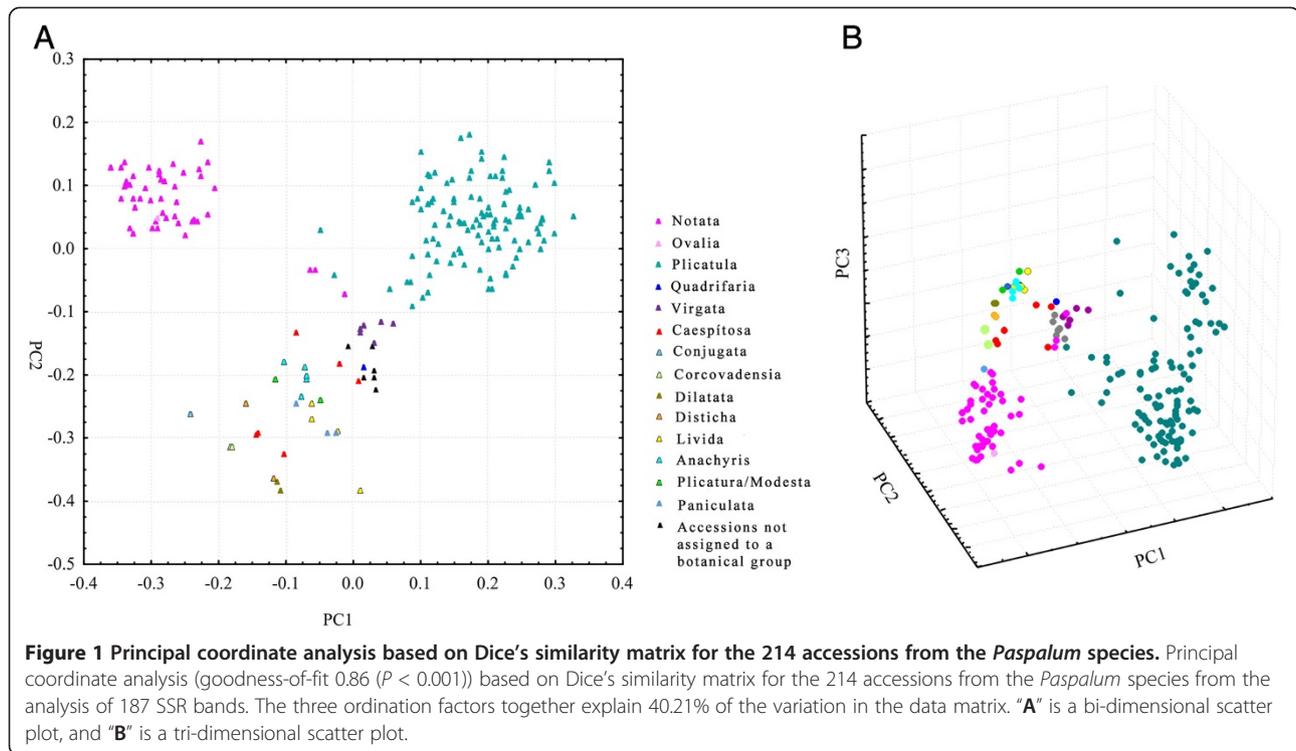
The PCO showed a goodness-of-fit value of 0.86 ( $P < 0.001$ ) and indicated the existence of three main groups (Figure 1), which correspond to the Plicatula group (except for some dispersed species) and the Notata group (*P. notatum*, *P. pumilum*, *P. subciliatum*, *Paspalum cromyorrhizon* Trin. ex Döll and *Paspalum ionanthum* Chase, with the last two being grouped more distantly from the other species in this group), and to a third group that is composed of other species from different botanical groups of *Paspalum* (Additional file 1). The three-dimensional PCO scatter plot (Figure 1B) showed the same trend of a tripartite division; however, the species of the Plicatula group were more dispersed. The three principal vectors, PC1 (26.77), PC2 (9.52) and PC3 (3.92), accounted for 40.21% of the variation. The NJ trees based on Dice's similarity coefficient support the results of the PCO analysis, identifying three main groups – the Notata Group, the Plicatula Group and a third group formed by species from other botanical groups (Figure 2).

The Bayesian analysis of the population structure using a model-based approach provided support for a genetic division of the *Paspalum* germplasm into three distinct groups (Figure 3), with  $K = 3$  (Additional file 5). Out of the twelve runs for  $K = 3$ , the run with the

**Table 2 Characteristics of the microsatellite loci from *Paspalum atratum* and *P. notatum* that were used in diversity analyses and cross-species amplification tests**

Locus name	Repeat motif	Ta (°C)	<i>Paspalum</i> genetic characterization			<i>P. notatum</i> genetic characterization			Reference/GenBank accession no.
			Product size (bp)	NM	PIC	Product size (bp)	NM	PIC	
PA02-A5	(GC)3GG(CT)4	60	122-142	9	0.59	NA	NA	NA	58
PA02-B1	(CGGC)3	60	130-202	23	0.81	158-170	5	0.53	58
PA01-B7	(GC)6(CA)2(GA)8CA(GA)9	60	185-310	32	0.89	249-300	7	0.68	58
PA01-C1	(CA)3CG(CA)2CT(AGGA)3	58	231	10	0.25	NA	NA	NA	58
PA02-C3	(CA)3CTGA(CA)4	60	222-400	13	0.78	240-266	3	0.09	58
PA01-C8	(AT)2TTA(AT)2TTCA(GA)2	60	247-279	6	0.76	NA	NA	NA	HM208140
PA01-E10	(GGT)2 T(ACT)2	54	186-210	6	0.43	NA	NA	NA	HM208141
PA02-F8	(AC)4TA(AC)3	TD	166-200	6	0.42	NA	NA	NA	58
PA01- F10	(CT)10	50	188-240	18	0.48	234-220	2	0.14	58
PN03-A6	(GT)8	60	152-192	20	0.76	160-188	11	0.86	57
PN02-B5B	(TCA)4	60	152-234	24	0.79	180-204	4	0.58	57
PN03-C7	(GT)6	50	133-177	20	0.83	159-165	3	0.58	HQ585425
PA02-G11	(CT)11	TD	*	*	*	NA	NA	NA	HQ585425
PA02-H1	(AC)3G(CA)3	TD	*	*	*	NA	NA	NA	HM208150
PA02-H4	(CA)2CT(CA)5 T(AC)2	TD	*	*	*	NA	NA	NA	58
PA02-H8	(GT)3GCA(TG)3	TD	*	*	*	NA	NA	NA	58
PN03-A5	(TG)8	60	*	*	*	249-287	4	0.54	57
PN03-F10	(TG)7	60	*	*	*	165-177	7	0.78	HQ585433
PN02-C6	(TG)8	51.4	*	*	*	139-145	4	0.29	HQ585422
PN02-F6A	(CT)11	60	*	*	*	154-218	22	0.89	57
PN03-D10	(GT)11	50	*	*	*	280-300	7	0.66	HQ585426
PN03-F2	(AC)7	60	*	*	*	241-249	5	0.64	57
PN03-F9	(CA)8	60	*	*	*	179-275	15	0.88	HQ585432
PN02-A12	(GA)3AT(GAA)3	60	NA	NA	NA	185-205	3	0.45	57
PN02-B3	(GT)12	50	NA	NA	NA	139-157	11	0.84	HQ585421
PN02-G10	(CA)8	60	NA	NA	NA	189-219	7	0.83	57
PN02-G3	(GT)9	60	NA	NA	NA	294-302	5	0.6	HQ585423
PN02-G7	(AC)7	TD	NA	NA	NA	118	7	0.74	HQ585424
PN02-H7	(AC)8	60	NA	NA	NA	194-204	4	0.63	57
PN03-D12	(AG)10	50	NA	NA	NA	220-240	7	0.69	HQ585427
PN03-E7	(GT)9	TD	NA	NA	NA	298	7	0.74	HQ585428
PN03-E9 <sup>b</sup>	(AC)7	60	NA	NA	NA	168-186	6	0.59	57
PN03-F1	(CA)7	50	NA	NA	NA	184-192	5	0.71	HQ585429
PN03-F3	(GT)8	60	NA	NA	NA	165-203	8	0.75	HQ585430
PN03-F7	(CA)10	50	NA	NA	NA	200-212	6	0.76	HQ585431
PN03-G5	(CA)7	60	NA	NA	NA	126-138	6	0.74	HQ585434
PN03-G8	(CA)7	60	NA	NA	NA	254-258	3	0.38	57
PN03-H10	(CT)11	60	NA	NA	NA	222-272	13	0.86	57
PN03-H3	(GA)22	51.4	NA	NA	NA	198-250	11	0.84	HQ585435
Average				15.58	0.65		7.52	0.64	

\* Test of cross-species amplification failed; NA: not analyzed; Ta: primer annealing temperature; TD: touchdown; NM: number of markers; PIC: Polymorphism information content.



highest likelihood value was selected to assign posterior membership coefficients (Q) to each accession, which supported the results obtained from the other methods of analysis, described previously (Figure 3).

The inter-species pair-wise genetic dissimilarity among the 214 accessions that belong to different *Paspalum* species varied from 0.00 among some *P. notatum* accessions to 0.80 among *Paspalum conjugatum* P.J.Bergius and *Paspalum rhodopedum* L.B.Sm. & Wassh., with an overall average of 0.43 being obtained.

#### Population structure in *Paspalum notatum*

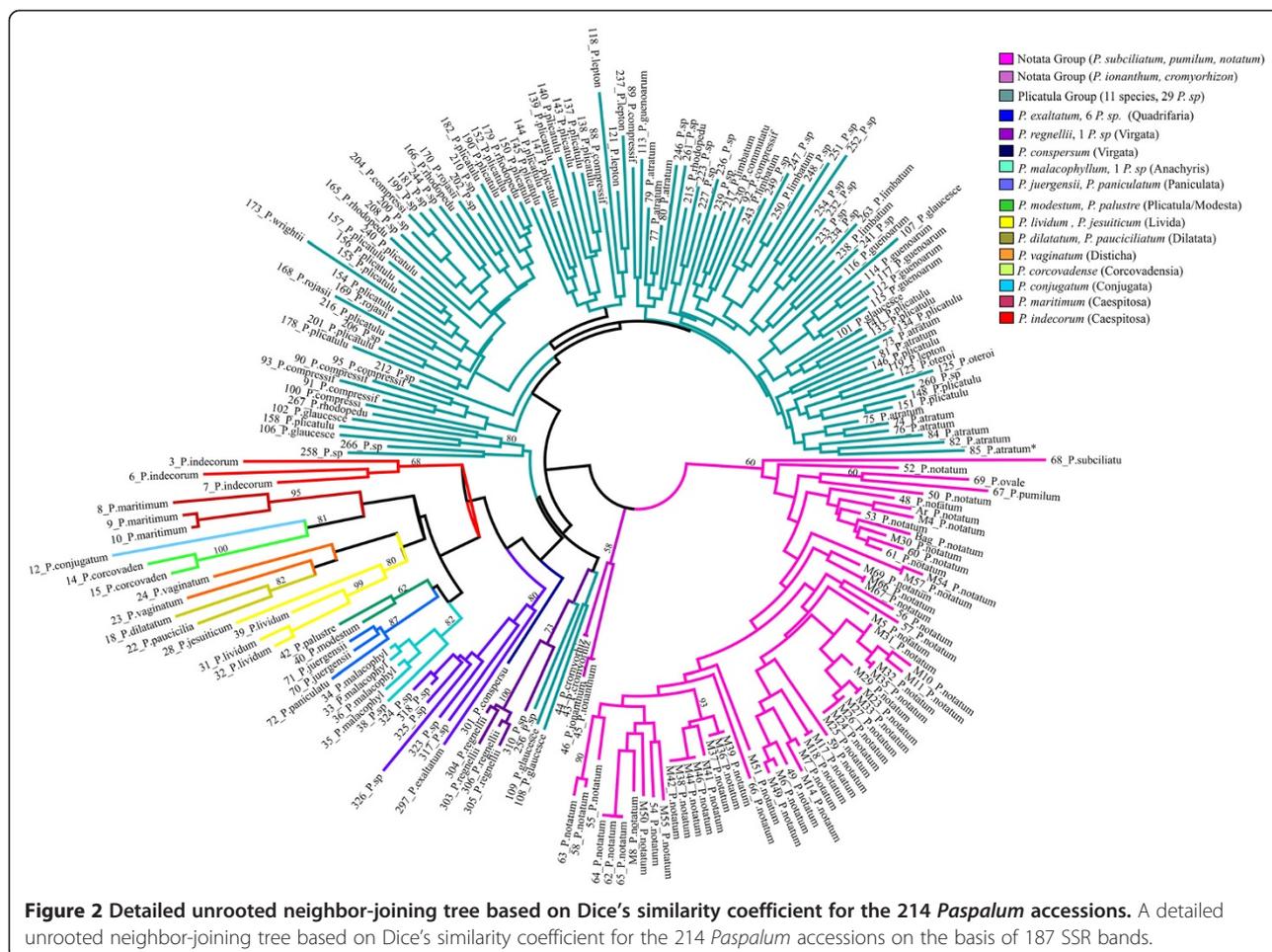
The Bayesian analysis performed with STRUCTURE software revealed two main values of K (Additional file 6). Figure 4 shows sub-figures for two K values up to the number of groups detected in UPGMA because both levels of subdivisions were instructive.  $K = 2$  separates individuals from S. Tome (Argentina) from the accessions from other localities, and the  $K = 7$  groups show correspondence to the botanical varieties of *P. notatum*. The accessions from groups A, B, C and D belong to the variety *notatum*, while the accessions from group E and some of the accessions from groups F and G belong to the variety *latiflorum*. The *saurae* variety accessions belong to the same genetic group (G). The number of accessions that correspond to each botanical variety and to each of the seven STRUCTURE groups with more than a 50% membership probability is shown in

Additional file 7. The M54 accession from the Guaíba region, RS, Brazil, showed a mixed group membership, which corresponded to both groups D (0.49) and G (0.487); however, this accession exhibits morphological traits that are more similar to group G. Group F corresponds to the accessions from S. Tome (Argentina), which separated when  $K = 2$ .

Similarity and distance-based methods (PCA, NJ, UPGMA) (Figures 5, 6, 7) revealed accession relationships that were consistent with the STRUCTURE-based membership assignment for most of the accessions (using  $K = 7$ , Figure 4), as indicated by the colors in the UPGMA dendrogram (Figure 6). On the other hand, the groups formed in PCA (Figure 7) indicate a correlation between the botanical varieties observed in *P. notatum*. The coefficients of similarity for all of the *P. notatum* accessions ranged from 0.17 to 1.0, with an average value of 0.42 being obtained. The cophenetic correlation coefficient in the UPGMA dendrogram was 0.92, which suggests that there were low levels of distortion between the matrix and the phenograms.

The AMOVA showed highly significant ( $P < 0.001$ ) differentiation among groups A, B, C, D, E, F and G, which indicates that approximately 47% of the observed variation can be explained by the subdivision of the accessions into these seven groups (Table 3).

In the NJ (not shown) and UPGMA trees (Figure 6), greater genetic divergence was observed among the



*saurae* variety accessions in comparison to other varieties, and *saurae* behaved as an outgroup in the dendrogram. This variety is distinguished from the others mainly by its ploidy level (diploid) and reproductive system (sexual self-incompatible), while *P. notatum* var. *notatum* and *P. notatum* var. *latiflorum* are either obligate or facultative tetraploid apomictics.

Exclusive bands were found between the different varieties (Table 4); however, these bands were not present in all of the accessions of a given variety but were instead differentially distributed between the corresponding gene groups.

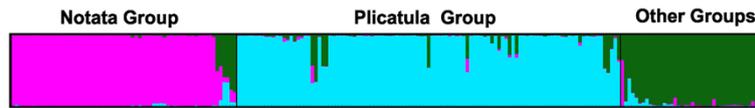
#### Genetic diversity and relationships among the seven groups of *Paspalum notatum* groups

Descriptive statistics for the *P. notatum* groups detected via STRUCTURE analysis (Table 5) revealed that groups E and G exhibited the highest genetic diversity. The overall  $\phi_{ST}$  among the seven groups was 0.47 (95% confidence interval,  $p < 0.001$ ), with the  $\phi_{ST}$  value for each cluster ranging from 0.46 to 0.52. Pairwise comparisons on the basis of the  $\phi_{ST}$  values can be interpreted as standardized population distances between any two groups.

The pairwise  $\phi_{ST}$  values obtained in this work ranged from 0.181 between groups E and G to 0.803 between groups B and F (Table 6). The genetic distance data agreed with the  $\phi_{ST}$  estimates. The smallest genetic distance was observed between groups E and G (0.222), whereas groups F and B presented the greatest genetic distance (4.075).

#### Morphological and molecular results from *P. notatum*

The PCA results indicated three main groups (Figure 7), which correspond to the three botanical varieties accepted by Parodi [36]. The features that contributed most to the morphological divergence between the groups were ALIB and LBL in principal component 1 (PC1) and SL and SW in principal component 2 (PC2), which together accounted for more than 55% of the detected variation. The group shown in dark green in the upper graph was botanically identified as var. *latiflorum* due to the size of its spikelets and the length of its inflorescence branches. However, the LBL and LBW were smaller than in other accessions that belong to var. *latiflorum*, which likely accounts for this difference. The results of the descriptive analysis (Additional



**Figure 3** STRUCTURE analysis for the 214 *Paspalum* accessions. A bar plot obtained from a model-based ancestry analysis of the 214 *Paspalum* accessions implemented within the STRUCTURE software.

files 8 and 9) and correspondence between informal *Paspalum* botanical groups and the STRUCTURE analysis groups (Additional file 10) are presented.

The correlation between the morphological traits and the SSR markers indicated a weak correlation between the morphological and molecular data ( $r = 0.42$ ;  $p < 0.001$ ).

#### Geographic distance and genetic dissimilarity in *Paspalum notatum*

The correlation between the geographic distance and the genetic similarity matrix was estimated for all the accessions and revealed no significant association between genetic and geographic variation based on  $r = 0.0017$  for the Mantel test (Additional file 11).

## Discussion

### Microsatellite markers

The *P. notatum* and *P. atratum* marker sets revealed polymorphisms in many of the other *Paspalum* species analyzed, which suggests that they are useful in genetic analyses of the *Paspalum* genus.

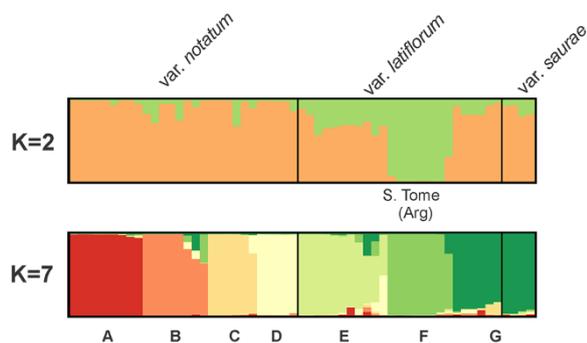
Some SSRs were highly polymorphic among the evaluated loci, and according to Huang et al. [92], the correlation between the number of bands revealed per locus and the PIC value is equivalent to Nei's gene diversity [93]. Testolin et al. [94] reported that highly polymorphic markers are promising for use in DNA fingerprinting to identify plant varieties or cultivars. We recommend that a subset of these loci be used in future fingerprinting studies, with a preference for loci that are easy to score

and that exhibit high heterozygosity, as indicated by high PIC values.

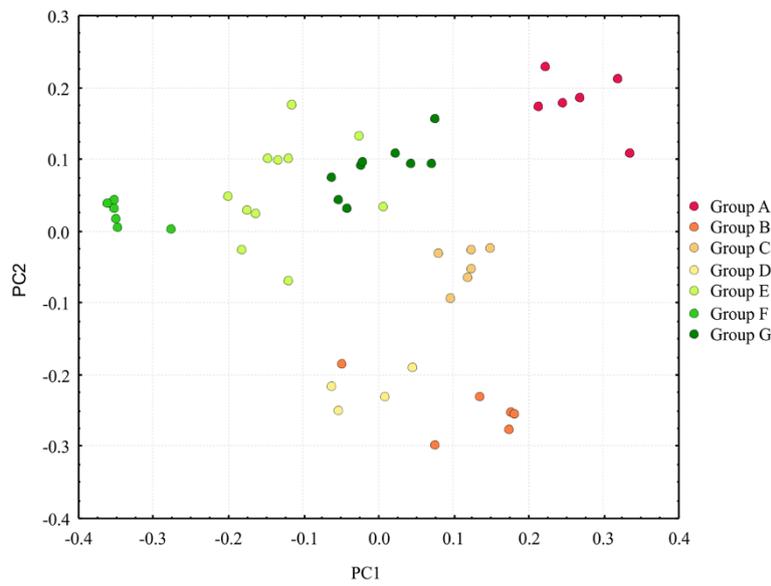
The genetic structure of the three genetic groups was confirmed using an AMOVA, where 46.83% of the variation was explained by the division into the three groups (Table 7). This finding suggests that the different species could contain unique alleles or genotypes and that these data can discriminate between taxa, especially the more distant taxa. Similar results were obtained by Jungmann et al. [47], who found that 44% of the variation in apomictic *Brachiaria humidicola* (Rendle) Schweick accessions could be explained by the subdivision of the germplasm into five groups.

The proportion of detected polymorphisms decreased as the genetic distance increased. This finding corroborates other results previously obtained in both plant [61,66] and animal studies [95]. We observed a reduced number of bands at some loci in species that were more distantly genetically related to the species for which the primers were designed (*P. notatum* and *P. atratum*). We suggest that this reduction might have been caused by the presence of null alleles or mutations that could have arisen in the primer-binding sites for the SSRs. Homoplasmy has been frequently detected among cross-species-amplified SSR markers [96], and it tends to complicate the interpretation of SSR variations. Non-amplifying or null alleles are not commonly found in plant species, but some examples of these phenomena have been observed [97]. The occurrence of null alleles could cause heterozygotes to be confused with homozygotes, which are usually inferred from significant heterozygote deficits. Undetected null alleles in populations can have dramatic effects on the interpretation of genotype frequency distributions and could lead to mistaken interpretations about the level of inbreeding in a population [98]. Therefore, care should be taken when using the studied loci to analyze species that are distant from the Notata and Plicatula groups, as they could lead to incorrect estimates. However, all of the examined loci were generally useful as an initial approach for the evaluation of *Paspalum* germplasm, especially for species that were more genetically distant from *P. atratum* and *P. notatum*.

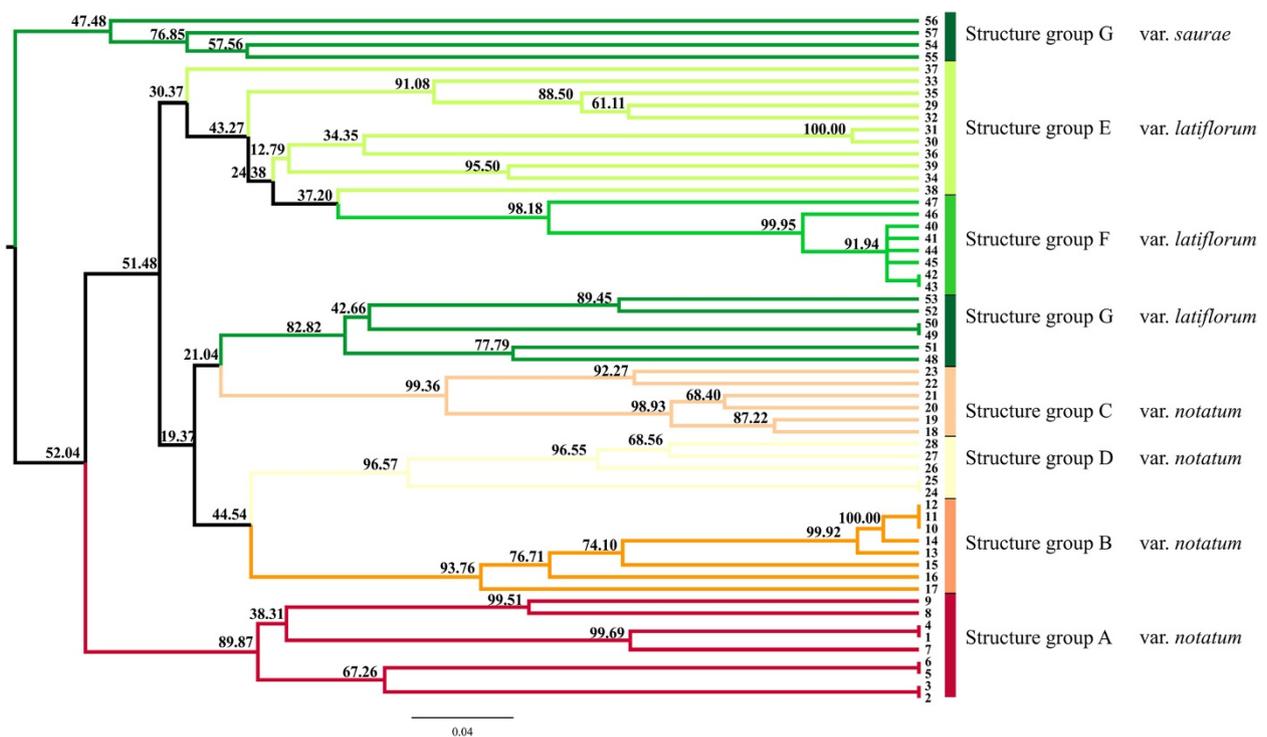
We observed the presence of exclusive bands between different varieties of *P. notatum*, which suggests that the use of these microsatellite markers can benefit taxonomic classification and breeding programs. These



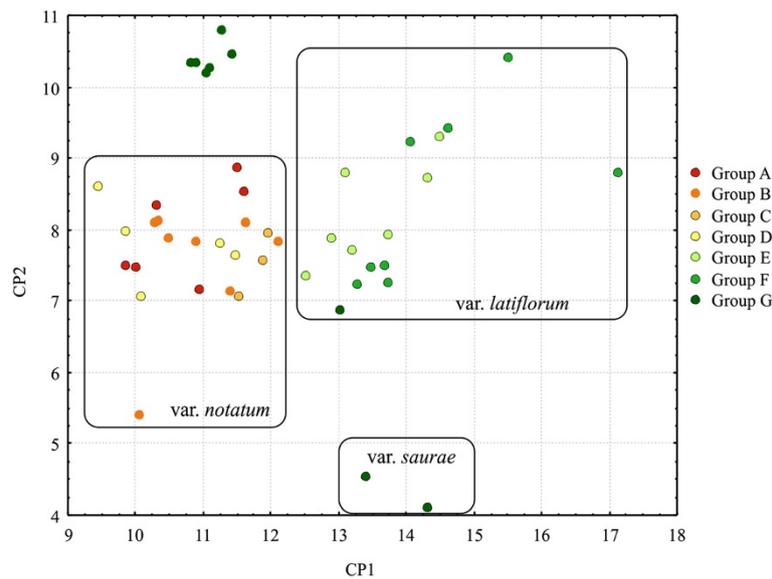
**Figure 4** STRUCTURE analysis of *P. notatum*. Structure analysis with  $K = 2$  and  $K = 7$  populations for the collection of *P. notatum* showing the sub-divisions within each variety, which are indicated.



**Figure 5** Principal component analysis based on Jaccard's similarity for *P. notatum*. PCA based on Jaccard's similarity, which resulted from 57 accessions of *Paspalum notatum*. The branch colors correspond to the colors of the STRUCTURE clusters from Figure 4, K=7.



**Figure 6** Dendrogram based on Jaccard's similarity coefficient for 57 *Paspalum notatum* accessions. An UPGMA dendrogram based on Jaccard's similarity coefficient for 57 *Paspalum notatum* accessions. The branch colors correspond to the colors in the STRUCTURE clusters from Figure 4, K=7.



**Figure 7** Principal component analysis based on nine morphological traits for *P. notatum*. A principal component analysis was based on nine morphological traits in 46 *Paspalum notatum* accessions. The branch colors correspond to the colors in the STRUCTURE clusters from Figure 4, K=7.

bands were not present in all the representatives of the same variety but were shared between accessions from the same gene pool. More detailed morphological and molecular characterizations of these groups should be performed to determine whether these characteristics are related to specific cultivars or biotypes, which we could not detect in our work.

#### Cluster analysis in the *Paspalum* genus

Our data support the usefulness of SSRs as a tool for the identification of genetic diversity and global similarity among members of a species. Therefore, we adopted a phenetic approach, which evaluates the genetic similarities between individuals.

All of the analyses identified three main groups (Figures 1, 2 and 3). This trend toward the formation of three main groups could have resulted from the low representative numbers of some species and groups as well as the nature of the markers used. These markers tended to be more conserved in the more genetically distant species of taxa for which the primer sets were originally developed (*P. notatum* and *P. atratum*, from the Notata and the Plicatula groups, respectively).

In this study, the species of the Notata group that form the subgenus *Paspalum* are represented by five taxa (*P. pumilum*, *P. notatum*, *P. subciliatum*, *P. cromyrorhizon* and *P. ionanthum*). The former two species were clearly distinguishable at the molecular level, whereas *P. pumilum*, *P. notatum*, *P. subciliatum* were grouped together. These results are in accordance with phylogenetic studies based on internal transcribed sequences (ITS) and chloroplast DNA (cpDNA), which support a close genetic relationship between *P. pumilum*, *P. notatum* and *P. subciliatum* [99-101] (these species form a monophyletic clade). Moreover, whether *P. cromyrorhizon* and *P. ionanthum* should be included in the subgenus *Paspalum* remains uncertain, but evidence provided by cpDNA sequences [99] supports a close relationship between these two species. *P. pumilum*, which is a sexual autogamous diploid species, showed less genetic divergence from *P. notatum* when compared with other species from the group. *P. subciliatum* exhibited a relatively intermediate degree of genetic divergence from the other species in the Notata group, whereas *P. cromyrorhizon* and *P. ionanthum* were classified as being somewhat more distant from the other group members (Figures 1, 2 and 3).

**Table 3** AMOVA for the groups of *Paspalum notatum* accessions detected via STRUCTURE analysis of 30 SSR markers

Source of variation	D.f.	Sum of squares	Variance of variation	Percentage
Among clusters	6	768.87	13.94 Va	47.05
Within clusters	50	784.66	15.70 Vb	52.95
Total	56	1,553.53	29.64	

**Table 4 Exclusive bands revealed by SSR markers in *Paspalum notatum* varieties**

SSR loci	Exclusive bands in <i>P. notatum</i> varieties		
	var. <i>notatum</i>	var. <i>latiflorum</i>	var. <i>saurae</i>
PN03-A6		bands 1, 10	
PN03-D12	band 6	bands 1, 4, 5 (240, 228, 226 bp)	
PN03-E7	band 7	band 1	
PN03-E9		band 3	
PN03-F2		band 5	
PN03-F3	bands 7, 8 (169, 165 bp)	band 6 (197 bp)	
PN03-F9	bands 1, 2, 3, 9 (275, 250, 248, 192 bp)	band 5 (220 bp)	
PN03-F10		band 1	
PN03-G3	band 1	band 5	
PN03-G7	band 7	band 1	
PN03-G8		band 1	
PN03-H3	bands 10, 11 (206, 198 bp)	bands 1, 9 (250, 210 bp)	
PN03-H10		bands 1, 2, 3, 5, 10, 11	band 13
PN02-B3		bands 7, 11 (145, 135 bp)	
PN02-C6		band 2	band 1
PN03-D10		bands 1, 2 (300, 296 bp)	
PN02-F6	bands 19, 20, 21, 22	bands 4, 5, 6, 7, 8, 9, 18	
PN02-G10		bands 1, 7	
PN02-H7	band 1		
PA02-B1	bands 1, 5 (170, 158 bp)		
PA01-B7		bands 1, 3, 6 (300, 288, 259 bp)	
PA02-C3		band 2 (242 bp)	band 1 (266 bp)

In this study, the Plicatula group showed high genetic variability within its species, but there was no clear distinction between different species (Figures 1, 2 and 3). The lack of a clear delineation between the different taxa of the Plicatula group is most likely caused by the genetic closeness of these species as well as the interspecific hybridization that has occurred throughout their evolution, which favors allele sharing. The complexity of the Plicatula was described in a taxonomic review of this group [22]. Many species of the Plicatula group are morphologically polymorphic, including *P. atratum*, *Paspalum plicatulum* Michx., *Paspalum compressifolium* Swallen, and *Paspalum limbatum* Henrard. Oliveira [22] suggested that the current circumscription adopted for

**Table 5 Descriptive statistics for the entire group of *Paspalum notatum* accessions and for the groups detected via STRUCTURE analysis of the 30 SSR markers**

Statistic	Overall	A	B	C	D	E	F	G
Sample size	57	9	8	6	5	10	8	11
Total number of alleles	208	102	91	94	83	146	84	144
Number of alleles per locus	6.93	3.40	3.03	3.13	2.77	4.87	2.80	4.80
Number of polymorphic sites	200	80	52	50	43	136	30	124
Genetic diversity (He)	0.68	0.55	0.54	0.53	0.52	0.65	0.51	0.65
PIC	0.64	0.50	0.48	0.48	0.44	0.60	0.45	0.60
Fst	0.47	0.46	0.50	0.49	0.49	0.43	0.52	0.43

\* A, C, D and E belong to the variety *notatum*; B, F and G, in general, belong to *latiflorum*; and F also includes var. *saurae*; Fst is the fixation index.

the separation of some species of the Plicatula group could include different taxa and that the pattern of evolution in the group is reticulated, with no well-defined morphological boundaries occurring between taxa. Although the species of this group have not been clearly delineated, the species of the Plicatula group were consistently grouped together in our analyses, with few exceptions. Polyploidy and hybridization are strategies that have played a role in the evolution of the species of the Plicatula group, as in most species of *Paspalum*, and should be accounted for when defining a species in this group. The species comprising the Plicatula group were considered to be those with populations that were formed by different co-specific cytotypes with at least a basic polyploid series of  $2n = 20$  or  $40$  [22].

Few conclusions could be made regarding the third main group, because it was composed of species from the other *Paspalum* botanical groups that were not included in the first two groups because of the small number of representative species. Previous molecular phylogenetic studies [99] have suggested a greater genetic distance between the third main group compared to

**Table 6 Genetic distances between the *Paspalum notatum* groups based on STRUCTURE analysis**

	A	B	C	D	E	F	G
A	0	0.54	0.48	0.51	0.28	0.70	0.39
B	1.16	0	0.62	0.60	0.40	0.80	0.44
C	0.92	1.61	0	0.61	0.31	0.77	0.39
D	1.03	1.49	1.54	0	0.31	0.75	0.38
E	0.38	0.66	0.46	0.45	0	0.47	0.18
F	2.31	4.07	3.35	2.96	0.90	0	0.42
G	0.64	0.80	0.64	0.60	0.22	0.72	0

The top diagonal is the pairwise  $\Phi_{ST}$ , and the bottom diagonal is the Slatkin distance. \* A, B, C and D belong to the variety *notatum*; E, F and G, in general, belong to *latiflorum*; and F also includes var. *saurae*.

**Table 7 AMOVA for the Paspalum accessions based on 187 SSR bands**

Source of variation	d.f.	Sum of squares	Variance components	% variation	P values
Among species	35	1296.14	5.67 Va	46.83	*
Within species	179	1132.83	6.44 Vb	53.17	*
Total	214	2428.97	12.10		

\*Significant difference with  $P < 0.05$ .

the Plicatula and Notata groups, which explains the lower frequency of microsatellite polymorphisms observed for species from the third group. However, there was a clear distinction between the species of this group. Thus, the markers employed in this study have proven to be effective in distinguishing the different *Paspalum* species and can assist in the identification of distinct species when combined with other molecular techniques.

The accessions from subgenus *Anachyris* were grouped and presented a close genetic similarity to the species from the Paniculata group (*Paspalum juergensii* Hack. and *Paspalum paniculatum* L.) (Figures 2 and 3). Our results corroborate the monophyly of the subgenus *Anachyris* [99] and the existence of close genetic relationships between *Anachyris* and *Paspalum falcatum* Nees ex Steud. (Falcata), *Paspalum paucifolium* Swallen (Eriantha), *Paspalum humboldtianum* Flügge (Subgenus *Ceresia*), *Paspalum quarinii* Morrone and Zuloaga (Quadrifaria), *P. juergensii* and *P. paniculatum* (Paniculata).

The Virgata and Dilatata groups represent species complexes that have been extensively studied and have been shown to be entirely composed of allopolyploid species based on cytogenetic evidence [27,102-112]. *Paspalum conspersum* Schrad., *P. dilatatum* and *Paspalum pauciciliatum* (Parodi) Herter exhibit genomic formulae of III<sub>2</sub>J<sub>2</sub>, IIIJX and IIIJ, respectively (Additional file 3). The donor species for genome I is *Paspalum intermedium* Munro ex Morong (Quadrifaria group), and the donor species for genome J is *P. juergensii* (Paniculata group). Although the Dilatata group has the genomic formulae IIIJ (Additional file 3), the two species from this group included in this study were not genetically close to the Quadrifaria and Paniculata groups, which include the donor species for genomes I and J, respectively.

The classification of *Paspalum* into two informal groups is controversial. *P. conspersum* (Virgata) and *Paspalum exaltatum* J. Presl (Quadrifaria) clustered together in the UPGMA dendrogram (Figure 6) and were shown to be genetically related to *Paspalum regnellii* Mez (Virgata). The genetic proximity of these species corroborates previous phylogenetic studies [99,113]. The Virgata group should be considered monophyletic, and a

number of additional species, such as *P. exaltatum*, should be included in it [113]. Moreover, their data supported the idea that *P. regnellii* should be excluded from the Virgata group and included in the Macrophylla group.

*Paspalum maritimum* Trin. (Caespitosa) is considered to be allopolyploid, with a predominant bivalent chromosome pairing [114], and this species clustered close to *P. conjugatum* and *Paspalum corcovadense* Raddi. *P. conjugatum* and *P. corcovadense* were grouped on the basis of the dendrogram and were shown to be genetically distant from the other species studied, corroborating previous phylogenetic studies [99].

#### Diversity and population structure in *Paspalum notatum*

Population structure analysis using STRUCTURE software corroborates the most supported groups found in the UPGMA analysis. Group F is the most consistent group throughout all the analyses, and it corresponds to all the accessions from S. Tome (Argentina) (Figures 4, 5 and 6). The  $K=7$  analysis confirmed the differences among the genotypes that are dispersed throughout southern South America (Additional file 11). The methodology used to analyze the molecular data in this work has been successfully employed for understanding how genetic diversity is organized in other economically important crops [47,115-117].

Populations of predominantly apomictic grasses are predicted to exhibit low levels of within-population genetic variation due to founder effects and a lack of recombination in offspring, whereas high levels of differentiation among populations are expected because of the limited gene flow via pollen and the divergence of populations over time caused by selection, drift and the accumulation of new mutations [118]. Our work focused on evaluating the relationships between accessions, which could represent distinct populations. Consequently, we expected to detect a high level of differentiation between individuals due to reproductive and geographic isolation. However, a high level of differentiation between individuals was not observed for all the accessions, such as the accessions from gene pools C and D, which are widely distributed but are genetically close. Furthermore, some accessions showed a large amount of genetic variation, especially those derived from Rio Grande do Sul (Brazil) and Argentina. The studied species is widely distributed in the Americas, and Argentina is considered to be its center of origin [119]. The most widely distributed accessions of *P. notatum* most likely originated from a few apomictic individuals who were better adapted to different environmental conditions, as reported for *Pennisetum setaceum*, an apomictic grass that has an invasive capacity and can colonize large areas with only a single clone [120].

Moreover, *P. notatum* comprises sexual diploid and facultative apomictic tetraploid individuals, and diversity

could be especially high in the apomictic species complexes that are derived from multiple, independent hybridization events. Gene flow in this species occurs between tetraploid and diploid cytotypes when they are in sympatry [44]; thus, diploid commercial cultivars can introduce variability into an apomictic complex. Furthermore, facultative apomictic tetraploid species can retain residual sexuality such that their occasional hybridization and genetic recombination introduces variability into natural populations. The better-adapted genotypes are then fixed via apomixis, which maintains the maternal genotype and heterozygosity through successive generations [121].

#### **Morphological and molecular results in *P. notatum***

The great morphological variability of *P. notatum* in Brazil has been previously described [7,37], and the morphological and genetic data reported here confirm these findings. Our genetic analyses show a weak correlation with phenotypic data, possibly as a consequence of the small number of morphological characters that we were able to use in this work, combined with the phenotypic plasticity of the morphological characteristics, which were mainly vegetative. Our results show that there is a tendency toward clustering the *P. notatum* accessions following the botanical variety classifications; however, our data could not confirm the informal intraspecific categories proposed by other authors because the morphological features employed to make these distinctions are continuous and often overlapping or are otherwise incapable of reflecting the total observed variation.

Using spikelet size as a diagnostic characteristic, Döll [41] described the variety *latiflorum*, which features spikelets that are orbicular-elliptical in shape and larger than the spikelets of the *notatum* variety. *Paspalum notatum* var. *latiflorum* Döll was previously accepted and cited by some authors [36,42,43], but this classification is not currently regarded as valid. Parodi [36] studied the variety *latiflorum* and proposed a new variety, *P. notatum* var. *saurae*, which is accepted and used to this day. To differentiate these varieties, Parodi [36] employed spikelet size and the length and number of inflorescence branches, which are diagnostic characteristics that exhibit little or no phenotypic plasticity. The morphological and molecular evidence obtained in this study supports the division of the species into three varieties because there is a strong tendency for accessions of the same variety to remain grouped into similar gene groups. Out of the seven distinct gene pools identified using Bayesian STRUCTURE software analysis, four represent the botanical variety *notatum*, and three pools correspond to the *latiflorum* variety.

Sexual diploid strains grow only in a limited area in Argentina, which is considered to be the center of origin for *P. notatum* [44,45]. Some tetraploids that share the

diploid gene pool might have maintained the genetic patterns of the Argentine diploid populations or might have arisen via independent self-polyploidization events. The diploid accessions (*P. notatum* var. *saurae*) were grouped into a single gene pool, which suggests that they share a common origin. These accessions could have derived from the cultivar Pensacola, which originated in Argentina [44,119], which is in agreement with the existence of gene flow between tetraploid and diploid cytotypes of *P. notatum* when they are sympatric [44]. However, a group of tetraploid accessions from western Rio Grande do Sul (Brazil) and Uruguay that were botanically identified as *P. notatum* var. *latiflorum* share the same gene pool with *P. notatum* var. *saurae*.

According to Quarín [24], autotetraploid species of *Paspalum* might have appeared in a two-step process. Crossbreeding sexual diploids sometimes develop aposporous embryo sacs. Occasionally, an unreduced oosphere is fertilized by the reduced spermatid nuclei of a diploid, forming a triploid ( $2n, 2x + n = 3x$ ). The triploids then produce offspring that, when fertilized by diploid pollen, give rise to tetraploid ( $2n, 3x + n = 4x$ ) genotypes.

#### **Geographic distance and genetic dissimilarity in *Paspalum notatum***

Our data suggest that there is no correlation between genetic dissimilarity and geographic distance among the *P. notatum* accessions. This arrangement might occur due to an apomixis and polyploidy-induced buffer that restrains the genetic status of the species and promotes the fixation of genetic fitness over a wide range of environments [45]. Moreover, the incongruence between the genetic and geographic data might also be a consequence of anthropogenic dispersal, because *P. notatum* is a turf and forage grass that is widely sown commercially and can be accidentally dispersed by cattle or other grazing animals. Although no correlation between genetic and geographical data was found in our analysis, a tendency for accessions from the same region to share the same gene pool was evident (Additional file 11).

#### **Conclusions**

In summary, our molecular genetic approach using cross-species amplification was proven to be useful for distinguishing the different taxa of *Paspalum* that were examined, with the exception of those that belong to the complex Plicatula group. The use of a molecular genetics approach that employs microsatellite markers in an initial assessment of germplasms was shown to be useful in species identification and in evaluating the possibility of successful species hybridization.

The methodological approach applied in this study allowed us to understand the genetic organization of different *P. notatum* genotypes. Comparing the genetic

organization with the morphological characteristics provided evidence that supports the existence of three botanical varieties (*P. notatum* var. *notatum*, *P. notatum* var. *saurae* and *P. notatum* var. *latiflorum* [36]), which are represented by different gene groups, with few exceptions. However, the wide genetic variation within each variety deserves more detailed study. We suggest using the methodology that we have proposed in this work to aid in species classification, especially for plants that exhibit wide morphological variation and are difficult to identify. In addition, the microsatellites developed and used throughout this work can be employed for taxonomic classification, breeding programs and DNA fingerprinting for *P. notatum* cultivar identification.

## Additional files

**Additional file 1: Accessions from the *Paspalum* collection used in the whole germplasm analysis.** Accessions from the *Paspalum* collection used in the whole germplasm analysis. The ID, BRA code, species, botanical groups, origin, chromosome numbers and the reference for the chromosome number determination are shown [23,31,32,114,122-126].

**Additional file 2: *Paspalum notatum* accessions evaluated in this study.** *Paspalum notatum* accessions evaluated in this study. The CODE used in the figures, ID, collector identification, BRA CODE, site of origin, chromosome number, ploidy level, geographic coordinates and availability of morphological data are shown.

**Additional file 3: Chromosome number, polyploidy type, reproduction mode and genomes found in the *Paspalum* species.** Chromosome number, type of polyploidy, reproduction mode and genomes found in the *Paspalum* species based on the literature [17,27,30-32,102-104,106-108,110,113,114,122,124,125,127-157].

**Additional file 4: Size range, number of bands and PIC from the 12 SSR loci transferred to the *Paspalum* taxa.** Size range, number of bands and PIC from the 12 SSR loci transferred to the *Paspalum* taxa.

**Additional file 5: Magnitude of  $\Delta K$  from STRUCTURE analysis of the germplasm.** Magnitude of  $\Delta K$  from STRUCTURE analysis of K (arithmetic mean  $\pm$  s.d. over 10 replicates) calculated following the  $\Delta K$  method proposed by Evanno et al. [84] for *Paspalum* microsatellite data. The modal values of these distributions indicate that the true K, or the uppermost level of the STRUCTURE analysis, is seven genetic groups.

**Additional file 6: Magnitude of  $\Delta K$  from the STRUCTURE analysis of the *Paspalum notatum* accessions.** Magnitude of  $\Delta K$  from the STRUCTURE analysis of K (mean  $\pm$  SD over 8 replicates), calculated following the  $\Delta K$  method proposed by Evanno et al. [84] for *Paspalum notatum* microsatellite data.

**Additional file 7: Mean probabilities for each population from the STRUCTURE analysis for K = 7 for *Paspalum notatum*.** Mean probabilities for each population from the STRUCTURE analysis for K = 7 for *Paspalum notatum*.

**Additional file 8: Statistics describing nine morphological traits from 46 *Paspalum notatum* accessions.** Statistics describing nine morphological traits from 46 *Paspalum notatum* accessions.

**Additional file 9: Principal components of nine morphological traits from 46 *Paspalum notatum* accessions.** Principal components of nine morphological traits from 46 *Paspalum notatum* accessions.

**Additional file 10: Botanical identification of *Paspalum notatum* accessions according to different authors and the corresponding STRUCTURE group.** Botanical identification of *Paspalum notatum* accessions according to different authors and the corresponding STRUCTURE group.

**Additional file 11: Map of the geographic distribution of 57 of the *Paspalum notatum* accessions analyzed.** Geographic distribution of the 57 *Paspalum notatum* accessions that were analyzed genotypically and phenotypically. Point colors correspond to the colors in the STRUCTURE clusters from Figure 5. A sole accession from Florida, USA, has not been represented in the map.

## Abbreviations

ALIB: Average length of inflorescence branches; AMOVA: Analysis of molecular variance; CTAB: Cetyltrimethylammonium bromide; GS: Genetic similarity; ISL: Inflorescence stem length; LBH: Leaf blade hairiness; LBL: Leaf blade length; LBW: Leaf blade width; LSL: Leaf sheath length; LSW: Leaf sheath width; PCA: Principal component analysis; PCO: Principal coordinate analysis; PCR: Polymerase chain reaction; PIC: Polymorphism information content; RS: Rio Grande do Sul state; SL: Spikelet length; SP: São Paulo state; SSR: Simple sequence repeat; SW: Spikelet width; UPGMA: Unweighted Pair Group Method with Arithmetic Mean; USA: United States of America.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

FWC performed experimental and statistical analyses and drafted the manuscript. MIZ participated in the statistical analysis. FHDS, JFMV and MD participated in the germplasm selection and the design and implementation of the study. APS and TTSC conceived the study and participated in its design and coordination. APS and BBZV helped draft the manuscript. All authors read and approved the final manuscript.

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