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Characterization of *Paspalum* genotypes for turfgrass cultivars development

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Abstract

The characterization of genetic resources is essential to carry out a breeding program. This study aimed to characterize *Paspalum* genotypes with potential use as soil surface covering to support breeding programs on the development of turf cultivars. Forty-three *Paspalum* genotypes, comprising 11 species, were evaluated. The embryo-sacs structure was determined by cleared ovaries analysis and eight accessions were classified as sexual, 15 as apomictic, and 20 presenting facultative apomixis. Most of the genotypes have 40 chromosomes, with the exception of one accession of *Paspalum vaginatum*, two of *Paspalum indecorum*, one of *Paspalum modestum*, and two of *Paspalum notatum* that have 20 and one accession of *Paspalum jesuiticum* and one of *Paspalum mandiocanum* that have 60 chromosomes. DNA content was determined by flow cytometry, ranging from 1.35 to 4.00 pg of DNA, with most of the accessions corresponding to tetraploidy, but also diploidy and hexaploidy were found, corroborating chromosome counts. High genetic variability was found among the 43 accessions based on 11 microsatellite markers and their use to estimate Jaccard similarity coefficients and Bayesian analysis, forming six different genetic groups. Considering only the *P. notatum* accessions, great variability was observed with four distinct groups formed. The results presented in this work reveal the possibility to obtain assertive crosses between compatible parents, aiming to explore the genetic variability between and within species of this genus.

1 | INTRODUCTION

Lawns are part of urban landscapes, accomplishing several environmental benefits, such as lowering the local temperature, carbon sequestration, soil erosion control, and air purification (Casler, 2006; Castro et al., 2015). In many countries, turfgrass farming encompasses billions of dollars and hundreds of cultivars. Turfgrass cultivars are developed for different uses, requiring specific characteristics, according to the purpose, for example, recreational and sportive lawns, soil covering, railway side, roadside, or airport runway safety areas (Casler, 2006).

Abbreviations: BGP, germplasm bank identification; DP, discriminatory power; PIC, polymorphic information content; SSR, simple sequence repeat; UPGMA, unweighted pair group method with arithmetic mean.

Thirteen turf species are registered in Brazil (MAPA, 2023; Villas Bôas et al., 2020), essentially represented by warm-season grasses. The cultivated turfgrasses with stoloniferous growth in Brazil are represented by *Axonopus affinis* Chase and *Stenotaphrum secundatum* (Walter) Kuntze (Christians et al., 2016), while the rhizomatous cultivars grown in Brazil are all from *Paspalum notatum* Flüggé. Some species and their hybrids that present both rhizomes and stolons are also cultivated: *Zoysia japonica* Steud; *Zoysia matrella* (L.) Merr.; *Zoysia japonica* Steud. × *Zoysia tenuifolia* Willd. ex Thiele (*Zoysia pacifica*); *Cynodon dactylon* (L.) Pers. × *Cynodon transvaalensis* Burtt Davy; *Cynodon dactylon* (L.) Pers.; and *Paspalum vaginatum* Swarz (Christians et al., 2016; MAPA, 2023; Villas Bôas et al., 2020).

In the early 2000s, the Brazilian market for turfgrass was estimated at about $50.65 million and included an annual demand of 17,000 ha (Zanon, 2003). The sod production takes place in 21 Brazilian states; among these, the state of São Paulo stands out as the largest producer, accounting for 43% of national production (Zanon & Pires, 2010). In 2015, the sod production area in Brazil was estimated at 24,000 ha based on surveys carried out by the National "Grama Legal" Association and Agrabras (Antoniolli, 2015). Recently, the Grama Legal Association estimated that the production area in 2020 reached 25,000 ha, as driven by the use of sod on highways and at airports (Villas Bôas et al., 2020).

The Brazilian turf grass market is mostly composed of exotic species, where *Zoysia japonica* ("Emerald grass") has 74% of the market share, being cultivated as lawns in public, domestic, or industrial gardens (Zanon & Pires, 2010). Despite the great diversity of Brazilian flora, few cultivars are available based on native species that were developed for covering soil surfaces, such as gardens or functional areas like airports, industrial parks, and highways (Freitas et al., 2002; Castro et al., 2015). Considering the genus *Paspalum*, bahiagrass or "batatais grass" (*P. notatum* Flüggé var. *notatum*) is widely used for composing landscapes as it has desirable characteristics like rusticity with high tolerance for low soil fertility and water deficiency, fast lateral growth with rapid covering soil surface, resistance to trampling, and fast recovery rate after dry and cold season (Kissmann & Groth, 1997; Freitas et al., 2002). However, most of the lawns formed with batatais grass originate from illegal sod collected in degraded pastures areas, being harmful for the environment (Arigoni, 2012; Souza et al., 2020).

The improvement of apomictic warm-season grass is commonly carried out through the selection of ecotypes from the natural variability of the germplasm collections (Acuña et al., 2019). Although it is an easy and fast breeding method, the selection based only on the evaluation of the adaptive capacity of materials collected in nature can present finite results (Valle et al., 2009). Furthermore, the success of using this method will depend on the genetic variability or number of different apomictic ecotypes existing within the species (Vogel & Burson, 2004).

Core Ideas

- These are genotypes with valuable characteristics, such as stress tolerance or disease resistance, which can be used in crosses aiming to develop a new cultivar.
- Genetic breeding is the manipulation of an organism's genetic makeup to create offspring with desired traits.
- Lawn is a managed area of low-growing, dense vegetation, primarily grasses, designed for aesthetic or recreational purposes.
- Germplasm is like the genetic library of an organism, containing the instructions for its current form and the potential for future adaptations.
- Apomixis is a way for plants to reproduce seeds asexually.

Paspalum is an essentially Pan-American genus of tropical and subtropical grasses. It comprises about 330 species (Zuloaga & Morrone, 2005), 216 of which are found in Brazil (Valls et al., 2023). A genebank of *Paspalum* with 538 accessions, collected in different regions of Brazil and representing 60 species, is hosted at Embrapa Southeastern Livestock, located in São Carlos, SP, Brazil (Matta et al., 2023). The remarkable range in plant size, leaf length, and growth habit observed within the Embrapa's *Paspalum* genebank indicates potential for diverse applications, from forage production to both ornamental and functional lawn uses. Souza et al. (2020) reports on the use of Embrapa's *Paspalum* genebank for selecting accessions suitable for ornamental turfgrass development. Authors evaluated 34 accessions for various characteristics, including ground cover, establishment time, regrowth rate, mowing frequency, plant height, and ornamental quality. Through this comprehensive evaluation, the study identified materials with desirable properties for lawn applications and/or turfgrass breeding programs, and five accessions were selected and registered in Brazil as lawn cultivars: *Paspalum lepton* 'Chauá' (BGP297 [where BGP is germplasm bank identification], registration number [RN]-36198), *P. notatum* 'Tuim' (BGP026, RN-36197), *P. notatum* 'Aruaí' (BGP155, RN-36828), *P. notatum* 'Tiriba' (BGP295, RN-41186), and *P. notatum* 'Maritaca' (BGP318, RN-36330) (MAPA, 2023).

However, for accessions to be used effectively in breeding programs, detailed information is required about their reproductive systems, ploidy levels, and the genetic variability of the traits of interest. This information guides the crosses made

to generate segregating populations, from which superior plants can be selected according to the specific goals of the cultivar (Ortiz et al., 2013). Several reproduction mechanisms are described in *Paspalum*, both sexual and apomictic, as well as different levels of ploidy between and within different species (Ortiz et al., 2013).

This study aimed to characterize *Paspalum* accessions with potential use as soil surface covering in terms of reproductive system, DNA content, number of chromosomes, and molecular diversity, to support breeding programs. We expand on the previous evaluations performed by Souza et al. (2020) by including additional accessions from the same genebank, chosen for their shared growth habit and visual appeal, further diversifying the available germplasm for turfgrass development.

2 | MATERIALS AND METHODS

2.1 | Material used

Forty-three *Paspalum* accessions, comprising 11 species, were evaluated (Table 1 and Table S1). From the 43 *Paspalum* accessions analyzed, 28 were evaluated by Souza et al. (2020) for turfgrass development potential, focusing on characteristics like ground cover ability, establishment time, and regrowth rate. The remaining 15 accessions were visually identified for their ground cover potential and compact growth habit. All accessions are conserved at the Embrapa's *Paspalum* genebank, situated at 21°96′17″ S and 47°84′21″ W, with 856 m of altitude (msl).

2.2 | Reproductive mode characterization

Inflorescences were collected in the morning, right after the anthesis, according to the respective flowering time of each accession. The determination of reproductive mode, if sexual or apomictic, was performed using the cleared-pistil technique, proposed by Young et al. (1979), with minor modifications. About 150 flowers from each accession, recently opened, with turgid stigmas, were collected in the GB-Paspalum and fixed for 24 h in solution of 95% alcohol:distilled water:40% formaldehyde: glacial acetic acid; 40:13:3:3 v/v.

After fixation, the flowers were transferred to 70% alcohol and stored in a refrigerator until analysis. The ovaries were isolated and clarified, replacing the 70% alcohol with the following series of solutions: 85% alcohol, absolute alcohol, alcohol: methyl salicylate (1:1), alcohol: methyl salicylate (1:3), 100% methyl salicylate (twice), remaining 24 h in each solution.

A total of 100 ovaries were investigated for each accession in an Axiophot1 microscope (Carl Zeiss) through the differential interference contrast microscopy technique. According to Ortiz et al. (2013), the embryonic sacs were classified as apomictic "*Paspalum* type" when presenting an egg cell with one or two synergids beside it, a large central cell with two polar nuclei, and absence of antipodal cells, while the meiotic or reduced embryonic sacs were classified as sexual "*Polygonum* type," because they have one egg cell, two synergids, a large central cell with two polar nuclei, and a minimum of three antipodal cells located at the opposite pole.

2.3 | DNA content determination

The flow cytometry technique was used for DNA quantification. Young leaves of each plant were collected in the field and placed in a plastic bag with paper moistened with water until the analysis. For each accession, approximately 5 mg of leaves from each plant, together with the same amount of young leaf tissue from *Raphanus sativus* cv. Saxa (internal reference standard), were ground in a Petri dish containing 800 µL of lysis buffer 01 (LB01) buffer (0.45425 g tris(hidroximetil)aminometano [TRIS], 0.186125 g disodium ethylene diamine tetra acetate [NaEDTA], 0.0435 g Spermina, 0.29225 g NaCl, 1.491 g KCl, 250 µL Triton in 250 mL of distilled water, pH 7.5, and 0.11% v/v of β-mercaptoethanol) chilled to obtain a nuclear suspension (Doležel, 1997). The ground tissue was aspirated through two layers of gauze and the nuclear suspension was subsequently filtered through a 40-µm mesh. To the nuclear suspension, 25 µL of propidium iodide and 25 µL of RNase were added. For each sample, at least 10,000 cores were analyzed. The analysis was performed on a FacsCalibur cytometer (Becton Dickinson) and the histograms obtained on the Cell Quest software and analyzed on Flowing Software version 2.4.1 (Perttu Terho, Turku Center for Biotechnology; www.flowingsoftware.com). The nuclear DNA content (pg) was estimated by comparison to the *Raphanus sativus* standard used, in which the 2C (DNA content in a diploid zygote cell) amount is equivalent to 1.11 pg of DNA (Doležel et al., 1992; Greilhuber et al., 2007). The ploidy level based on DNA content was estimated by comparison to previous studies of other authors and to the results of chromosome counting of the accessions in this work.

2.4 | Chromosome number determination

The chromosome number of some accessions had already been determined in previous studies (Table 1). In this work, it was observed that the chromosome number of 15 accessions (BGPs: 114, 230, 273, 291, 295, 297, 304, 321, 325, 333, 340, 382, 394, 405, and 412). For meiosis characterization, samples of plant inflorescences were collected in the

TABLE 1 DNA content, chromosome number, and reproductive structure of 43 *Paspalum* accessions.

Species	BGP	M	A	M + A	E	RM	Chromosome number and reference	C
P. conjugatum P.J. Belgius	045	100	0	0	0	S	NE	2.00
P. conjugatum P.J. Belgius	382	75	4	0	21	FA	40	3.41
P. denticulatum Trin.	027	0	92	0	8	OA	40 (Adamowski et al., 2005)	2.08
P. denticulatum Trin.	033	0	96	0	4	OA	40 (Pagliarini et al., 1999, 2001)	2.13
P. denticulatum Trin.	235	0	100	0	0	OA	40 (Pozzobon et al., 2008)	2.50
P. indecorum Mez	046	100	0	0	0	S	20 (Burson, 1985; Fernandes et al., 1974; Pagliarini et al., 1999, 2001; Pozzobon et al., 2013; Quarin & Burson, 1983)	1.35
P. indecorum Mez	233	100	0	0	0	S	20 (Pozzobon et al., 2008)	1.37
P. jesuiticum Parodi	218	7	91	0	2	FA	60 (Bernardo Filho et al., 2014)	3.12
P. lepton Schult.	063	1	99	0	0	FA	40 (Adamowski et al., 2005; Pozzobon et al., 2000)	3.13
P. lepton Schult.	297	17	71	0	12	FA	40	3.04
P. lepton Schult.	321	0	100	0	0	OA	40	3.48
P. lepton Schult.	135	0	100	0	0	OA	40 (Pozzobon et al., 2000)	4.00
P. lepton Schult.	325	0	100	0	0	OA	40	3.42
P. lepton Schult.	394	4	96	0	0	FA	40	3.43
P. lepton Schult.	333	37	28	0	35	FA	40	3.23
P. mandiocanum Trin.	230	0	100	0	0	OA	60	3.20
P. modestum Mez	032	100	0	0	0	S	20 (Pozzobon & Valls, 2003)	1.52
P. notatum Flügge	022	100	0	0	0	S	20 (Pagliarini et al., 1999, 2001)	1.46
P. notatum Flügge	026	25	58	0	17	FA	40 (Pagliarini et al., 1999)	2.61
P. notatum Flügge	030	38	56	0	6	FA	40 (Pozzobon & Valls, 1997)	3.49
P. notatum Flügge	034	20	80	0	0	FA	40 (Adamowski et al., 2005; Pozzobon & Valls, 1997)	2.74
P. notatum Flügge	047	0	96	0	4	OA	40 (Pagliarini et al., 1999; Pozzobon & Valls, 1997)	3.22
P. notatum Flügge	115	0	95	0	5	OA	40 (Pozzobon & Valls, 1997)	2.93
P. notatum Flügge	155	61	34	0	5	FA	40 (Pozzobon & Valls, 1997)	3.54
P. notatum Flügge	214	28	57	15	0	FA	NE	3.32
P. notatum Flügge	216	100	0	0	0	S	40 (Forbes & Burton, 1961)	2.70
P. notatum Flügge	241	6	3	0	91	FA	40 (Dahmer et al., 2008)	3.28
P. notatum Flügge	242	9	91	0	0	FA	40 (Dahmer et al., 2008)	2.68
P. notatum Flügge	405	0	100	0	0	OA	40	3.31
P. notatum Flügge	371	3	97	0	0	FA	NE	2.43
P. notatum Flügge	291	0	100	0	0	OA	40	2.61
P. notatum Flügge	294	7	93	0	0	FA	40 (Souza-Chies et al., 2006)	3.32
P. notatum Flügge	295	80	19	0	1	FA	40	2.92
P. notatum Flügge	304	33	59	0	8	FA	40	3.31
P. notatum Flügge	306	100	0	0	0	S	20 (Dahmer et al., 2008)	1.70
P. notatum Flügge	318	0	92	8	0	OA	NE	2.73
P. notatum Flügge	340	12	83	0	5	FA	40	2.34
P. notatum Flügge	412	9	50	23	18	FA	40	2.44
P. notatum Flügge	414	0	100	0	0	OA	NE	2.31
P. oteroi Swallen	118	0	100	0	0	OA	40 (Pagliarini et al., 1999, 2001)	3.20

(Continues)

TABLE 1 (Continued)

Species	BGP	M	A	M + A	E	RM	Chromosome number and reference	C
P. oteroi Swallen	273	0	100	0	0	OA	40	3.29
P. subciliatum Chase	204	13	71	0	16	FA	40 (Adamowski et al., 1998; Pagliarini et al., 1999)	1.82
P. vaginatum	114	100	0	0	0	S	20	2.00

Abbreviations: A, single or multiple aposporic embryo sac; BGP, genebank accession identification number; C, DNA content in cells in picograms (pg of DNA); E, sterile embryo sac; FA, facultative apomictic reproduction; M, meiotic embryo sac; M+A, embryonic sac showing meiotic and aposporic structures; NE, not evaluated; OA, obligatory apomictic reproduction; RM, reproduction mode; S, sexual reproduction.

field and fixed for 24 h in a 3:1 solution (ethanol:acetic acid, v/v), then transferred to 70% alcohol and subsequently stored at 40˚C until analysis. The anthers were crushed and stained with 2% acetic carmine during the slides confection. For mitosis characterization, seedlings were removed from the plots and placed in plastic cups with water to induce rooting. Three days later, root tips were collected and placed in microtubes with bromonaphthalene for 2 h. Then, the roots were fixed in Carnoy's solution for 2 h. For staining, the roots were treated with 1N HCl in a water bath for 10 min and then stained with Schiff's reagent for 2 h. The roots were conserved in distilled water. For cell observation, anthers or root tips were macerated on a slide with 2% acetic carmine and observed under an optical microscope. Chromosomes were counted in premetaphase or metaphase.

2.5 | Molecular characterization

The DNA extraction from leaf samples was performed using the cetyltrimethylammonium bromide (CTAB) protocol, according to Doyle and Doyle (1987). Eleven simple sequence repeats (SSRs) loci developed for the species *P. notatum* and *Paspalum atratum* (Cidade et al., 2009, 2013) were selected, which were amplified by polymerase chain reaction according to Cidade et al. (2009). DNA fragments were visualized by electrophoresis in 6% polyacrylamide gels (weight/volume) and stained with silver nitrate, according to Creste et al. (2001), for polymorphism and genetic variability evaluation of the accessions. The bands were genotyped as presence or absence of homologous DNA and computed in a binary matrix once most of the genotypes are polyploid. The presence of a band, for one or more individuals of the same species, per locus, and their absence in the other analyzed species were considered to determine the private bands number. The polymorphic information content (PIC) (Cordeiro et al., 2003) was estimated using the online calculator "PIC calculator," (https://www.liverpool.ac.uk/~kempsj/pic.html). In addition, the discriminatory power (DP) of the adopted markers was calculated to compare their efficiency in the samples differentiation (Tessier et al., 1999).

Bayesian method for estimation of genetic population structure was applied to estimate the number of groups using the STRUCTURE v.2.3.4 software (Pritchard et al., 2000). All simulations were performed under the mixing model and correlated allele frequencies, with 500,000 burn-in iteractions and 800,000 repetitions for Markov chain and Monte Carlo processes. The optimal number of clusters was determined through the values of ln(k) and ΔK (Evanno et al., 2005), as implemented in STRUCTURE HARVESTER (Earl & von Holdt, 2012). The genetic distances between pairs of accessions were estimated using Jaccard's similarity coefficient (Jaccard, 1908), with the dendrogram construct based on the unweighted pair group method with arithmetic mean (UPGMA) method. These analyses were carried out in the NTSYS program (Rohlf, 1997).

3 | RESULTS

3.1 | The 43 *Paspalum* spp. accessions

Among the evaluated 43 *Paspalum* accessions, eight were classified as sexual, 16 as apomictic, and 20 accessions were presented as facultative apomixis, in which the same plant presents flowers with embryonic sacs of the *Paspalum* type and flowers with embryonic sacs of the *Polygonum* type (Table 1). The sexual accessions showed only *Polygonum*-type structures (Figure 1A). Sterile ovules were observed, that is, without embryonic sacs (Figure 1B) and, therefore, without reproductive structure inside. With regard to the apomictic accessions, single structures (Figure 2A) of the *Paspalum* type, or multiple structures were observed, with both structures, the *Polygonum* type and the *Paspalum* type (Figure 2B). In the latter case, apomixis will be the prevalent reproductive system (Hojsgaard et al., 2013).

Distinct reproductive systems were verified in different accessions of the same species. Accession *Paspalum conjugatum* BGP045 showed sexual reproductive structure, while accession *Paspalum conjugatum* BGP382 showed facultative apomixis. The same characteristics were repeated in the species *P. notatum* and *P. vaginatum*. Nonetheless, the

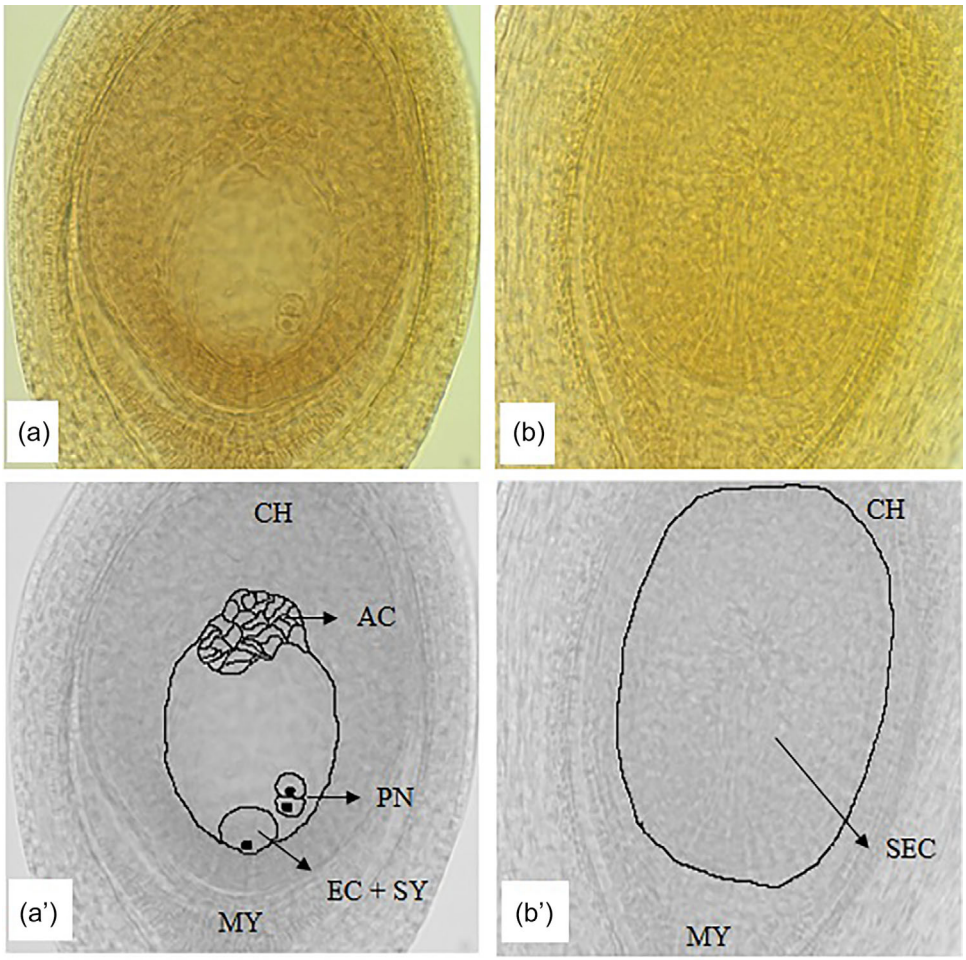

FIGURE 1 Cleared *Paspalum* ovules showing (a, a') polygonum-like meiotic structure of *Paspalum indecorum* (BGP233) and (b, b') sterile ovule, without embryo sac of *Paspalum notatum* (BGP241); (a') and (b') are the respective highlighted structures for better visualization. AC, antipodal cells; CH, chalaza; EC, egg cell; MY, micropyle; PN, polar nuclei; SEC, sterile embryo sac; SY, synergids.

accessions of *Paspalum indecorum* and *Paspalum modestum* presented only sexual reproductive structure, while *Paspalum denticulatum*, *Paspalum mandiocanum*, and *Paspalum oteroi* accessions presented only obligatory apomixis reproductive structure. The only evaluated accessions of *Paspalum jesuiticum* and *Paspalum subciliatum* were facultative apomictic, while *P. lepton* had both obligatory and facultative apomixis (Table 1).

The chromosome counting values for all accessions are shown in Table 1. Most accessions have 40 chromosomes, with the exception of one accession of *P. vaginatum*, two accessions of *P. indecorum*, one accession of *P. modestum*, and two accessions of *P. notatum* (BGP022 and BGP306) that have 20 chromosomes and one accession of *P. jesuiticum* and one accession of *P. mandiocanum* that have 60 chromosomes. The amount of DNA estimated among the 11 species of *Paspalum* ranged from 1.35 to 4.00 pg of DNA, with an average of 2.74. There was variation in the estimated amount of DNA among accessions belonging to the species *P. conjugatum*, *P. lepton*, and *P. notatum*.

The SSRs markers characterization for all 43 evaluated accessions identified 177 bands for the 11 analyzed markers, of which 43% were detected as exclusive in the species evaluated by the set of loci of the study, in addition to observing high values of PIC and DP (Table S2). The number of bands (NB) ranged from 8 (PN03-E9) to 23 (PN02-F6A). The used microsatellite markers showed high polymorphism among the evaluated accessions, with the PN03-E9 marker being the least informative and the PN02-F6A marker being the most informative, both for the total set of genotypes and only for the accessions belonging to the species *P. notatum*.

The Jaccard similarity coefficients among the 43 accessions ranged from 0.00 to 1.00, with a mean of 0.16 (Table S3). Through the methodology proposed by Evanno et al. (2005), a *K* value of 6 was estimated as the most likely number of clusters in Bayesian analysis (Figure S1A), which is shown in Figure 3. The UPGMA grouping pattern presented medium bootstrap values and did not present correspondence to the groups observed in the Structure analysis. Not all accessions of the same species were classified in a single cluster.

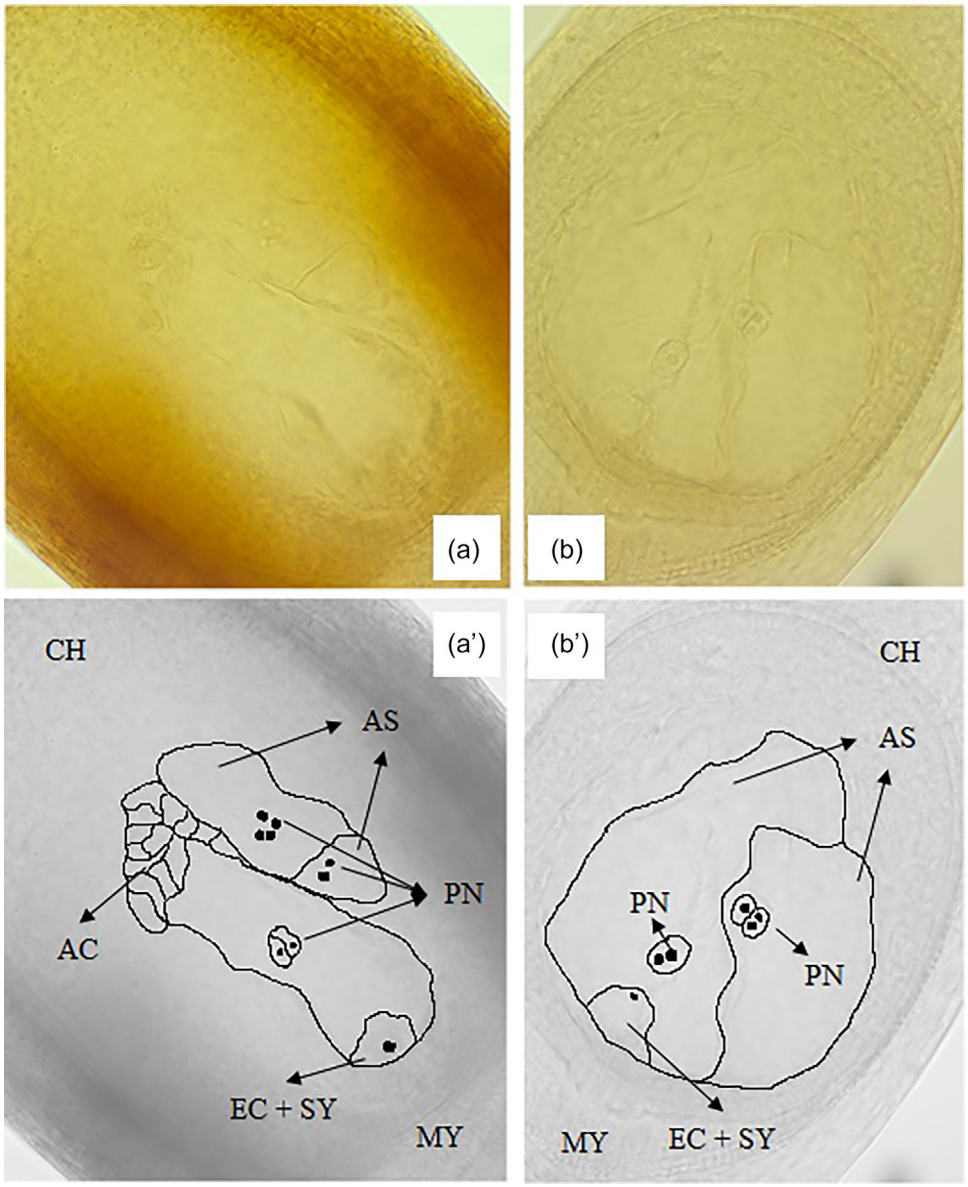

FIGURE 2 Embryonic sacs: (a, a') Multiple sacs, one aposporic and the other meiotic together, with apomictic reproduction (*Paspalum notatum* BGP214) and (b and b') multiple aposporic sacs, of the *Paspalum* type, with apomictic reproduction (*Paspalum denticulatum* BGP027). (a') and (b') are the respective highlighted structures for better visualization. AC, antipodal cells; AS, aposporic sacs; CH, chalaza; EC, egg cell; MY, micropyle; PN, polar nuclei; SEC, sterile embryo sac; SY, synergids.

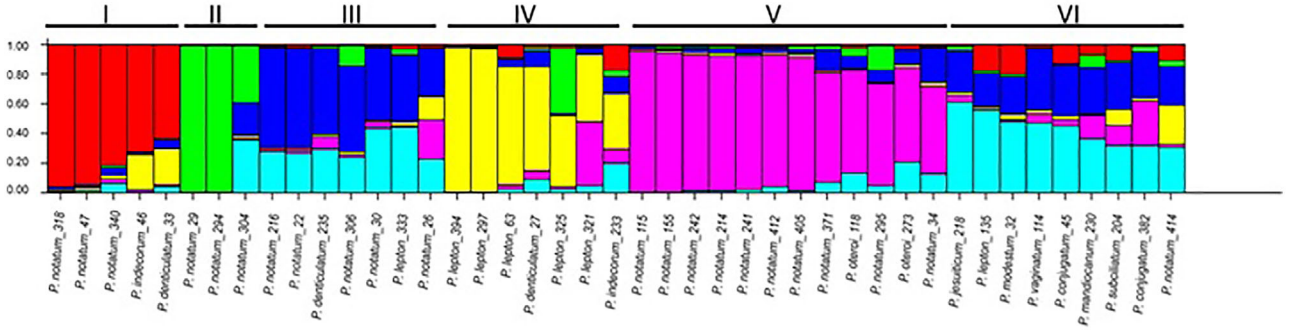

FIGURE 3 Grouping of the 43 accessions of *Paspalum* spp. based on six gene pools ($K = 6$; red, green, blue, turquoise, yellow, and lilac gene pools) from the Bayesian analysis. Species and genebank accession identification numbers are indicated below each sample.

P. notatum accessions were classified into five clusters; *P. lepton* was presented into three clusters and *P. denticulatum* into two clusters (Figure 3). The variability between individuals of the same species, based on only 11 SSR molecular markers, is highlighted by different colors of gene pools visualization detected through the Bayesian analysis.

3.2 | The 22 *Paspalum notatum* accessions

Regarding the reproductive systems, the accessions of *P. notatum* showed substantial variability. Most accessions showed tetraploidy and apomixis; sexuality was also found for two diploid accessions (BGP022 and BGP306) and one tetraploid accession (BGP216). Sterile embryo sacs were observed in varying degrees of occurrence among accessions, with accession BGP241 having 91% of sterile embryo sacs. In this context, it is worth reporting that DNA content estimates exhibited a notable range across the 22 examined accessions. The molecular characterization showed 124 bands for the same set of SSR markers, and the NB ranged from 6 (markers PN03-E9 and PN02-A12) to 14 (markers PN02-H7 and PN02-G3). High PIC and DP values were also observed for *P. notatum*, with PIC values always greater than 0.722 and DP values were greater than 0.848 (Table S2). Likewise, in this set of accessions, marker PN03-E9 was the least informative, while marker PN02-F6A was the most informative.

The Jaccard similarity coefficients for *P. notatum* ranged from 0.00 to 0.85, with a mean of 0.19 (Table S4). According to the methodology proposed by Evanno et al. (2005), the most probable K values were $K = 2$ and $K = 4$, in this order (Figure S1B). However, the two Structure groups formed with $K = 2$ were (data not shown) as follows: one group with the accessions BGP 291, 294, 295, 304, and 306, which correspond to the green genetic pool in Figure 4 (Group II + BGP 306), and the other group with the other 17 accessions. Based on this result, we decided to choose the second most probable K number (4), due to the better representation of the great genetic diversity observed and to the accordance between the Structure and UPGMA groups, which considers the separation between the Group I and the combination of Groups II, III, and IV that is highly supported by the bootstrap analysis in UPGMA (Figure 4) and is not considered in Structure with $K = 2$.

4 | DISCUSSION

4.1 | The 43 *Paspalum* spp. accessions

In this study, we evaluated 43 accessions from the *Paspalum* genebank of Embrapa, with potential for lawn applications. Twenty-eight of them were evaluated by Souza et al. (2020) in regard to specific traits desirable for the development of turfgrass cultivars.

Different reproductive strategies were detected among the accessions evaluated in this work, as well as variation in DNA content among the 11 evaluated *Paspalum* species. Jarret et al. (1995), when considering several species of *Paspalum*, reported DNA content values between 1.02 and 1.15 pg for diploid species, between 1.94 and 2.21 pg for tetraploid species, and 2C = 3.86 pg of DNA for a hexaploid accession of the species *Paspalum pubiflorum*. However, according to Vaio et al. (2007), these ranges can vary greatly when considering the different types of genomes within the genus *Paspalum*.

Although the apomixis reproduction mode of most of the accessions, inter- and intraspecific genetic variabilities were verified through the identification of six different gene pools in the molecular analysis and through the distinction of individuals that presented a mixture of these same pools. To illustrate, the apomictic accessions BGP033 and BGP235 of *P. denticulatum*, collected in the city of Uruguaiana-RS (Table S1), were classified into different gene pools (Figure 3). Moreover, the unexpected grouping pattern observed considering the *Paspalum* taxonomy (Figure 3) might be occurring due to (1) the use of nuclear microsatellite markers used are probably not linked to the reproductive characters that are used in *Paspalum* taxonomy, (2) the number of molecular markers used that are not enough to explain this great diversity and to its lack of capacity of differentiating all the accessions among each other, and (3) the low number of genotypes from each species for most of the species that hinders the identification of private alleles from a species, as observed for *Paspalum* by F. A. Oliveira et al. (2016).

The occurrence of sexual diploids and apomictic tetraploids within the same species was verified, as observed in *P. notatum* (see next Section) and *P. conjugatum*. The accession of *P. conjugatum* BGP045 showed sexual reproductive structure, in which all evaluated embryo sacs presented reproductive structure of the *Polygonum* type, and DNA content compatible with the level of diploid ploidy. The accession *P. conjugatum* BGP382 showed facultative apomixis and DNA content compatible with a tetraploid ploidy level. In this species, variations in the number of chromosomes have already been described, in different accessions, with variations of $2n = 20, 40, 60$, and 80 (Hojsgaard et al., 2009). Furthermore, these two accessions also showed a similar allelic constitution in Group VI (Figure 3), which does not prevent them from being crossed with each other. Sexual accession from other species has also been identified. The only evaluated accession of *P. vaginatum* (BGP114) was considered sexual, presenting a DNA content of 2.02 pg, corroborating to Liu et al. (1994). This result is according to the literature for the species, where only accessions with 20 chromosomes and sexual reproductive mode were found (Bashaw et al., 1970; Pozzobon et al., 2000). The only evaluated accession of *P. modestum* was sexual with

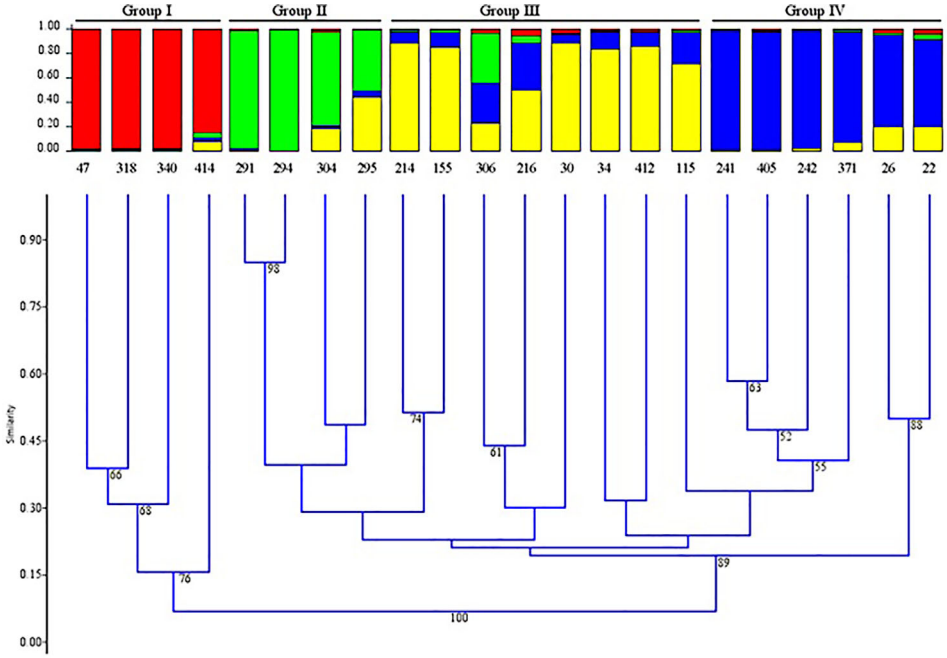

FIGURE 4 Unweighted pair group method with arithmetic mean (UPGMA) tree grouped by Jaccard's similarity coefficient for the 22 evaluated *Paspalum notatum* accessions and the Bayesian clustering pattern based on $K = 4$ (red, green, yellow, and blue gene pools) pointing out the genetic differences. Bootstrap values above 50% are indicated in the branches. Genebank accession identification numbers are indicated for each sample.

DNA content compatible with the diploid level, corroborating to Ortiz et al. (2013). The accessions of *P. indecorum* (BGP046 and BGP233) were considered sexual, also presenting 20 chromosomes and with DNA content compatible for diploid plants, corroborating with results of Pozzobon et al. (2013). Through the Bayesian analysis, the BGP046 and BGP233 accessions presented different allelic constitutions, showing variability between them (Figure 3).

The three evaluated accessions of *P. denticulatum* were collected in the state of Rio Grande do Sul (Table S1) and reproduced by obligate apomixis. Sartor et al. (2011) described three other accessions as facultative apomictic genotypes. Regarding the genetic background, the three evaluated accessions in this study have different genetic backgrounds (Figure 3), indicating high genetic variability among them. It is worth highlighting that Sartor et al. (2011) reported the identification of diploid and tetraploid sexual genotypes, which could be explored in crossbreeding programs.

P. jesuiticum and *P. subciliatum* were identified as facultative apomictic (a single accession of each species was analyzed). According to Bernardo Filho et al. (2014), this accession of *P. jesuiticum* has 60 chromosomes and the accession of *P. subciliatum* has 40 chromosomes (Adamowski et al., 1998; Pagliarini et al., 1999). Therefore, the observed reproduction mode is compatible with the polyploid character of the accessions. The accession of *P. mandiocanum* was identified as obligate apomictic with 60 chromosomes, as found by Burson and Bennett (1971). The accessions evaluated in the present work regarding DNA content had their results corroborated to Jarret et al. (1995) and Galdeano et al. (2016).

Facultative or obligate apomixis was observed in *P. lepton* accessions. As *P. lepton* is synonymous with *Paspalum nicorae* (R. C. Oliveira & Valls, 2008), several studies refer to this name. Thus, *P. lepton* is described in the literature as an apomictic tetraploid (Reis et al., 2008), although there are no studies about the percentage of apomixis in the species' genotypes. The values of C-DNA content and the number of chromosomes are consistent with the tetraploid condition described for *P. lepton*. The accessions BGP063, BGP297, and BGP394 of *P. lepton* showed similarity to each other, considering the genetic background characteristics, and they shared with BGP321 and BGP325 (all from Group IV) a good part of the yellow allelic pool. Although with a different constitution, the other two evaluated *P. lepton* accessions (BGP135 and BGP333) were similar to each other, despite being in different groups, as the allelic pools proportion has differed between them (Figure 3).

Hybrid turfgrass cultivars with improved traits can be developed through crosses between sexual and apomictic accessions, expanding on successful practices in forage breeding (Acuña et al., 2009, 2019). The genetic and reproductive characterization of *Paspalum* accessions in this study demonstrate the possibility of carrying out breeding programs based on hybridization and selection, supported by the substantial morphological variability of the genotypes of the *Paspalum* genebank. However, we must highlight

the necessity of using tetraploidized sexual individuals as female parents, considering the ploidy differences between sexual and apomictic accessions for crossing. Fortunately, there is a tetraploid sexual accession, represented by BGP216 (Q 3664), available in the genebank. Previous works report interspecific crosses between some *Paspalum* species evaluated here. Quarin and Burson (1983) successfully performed crosses between *P. indecorum* × *P. vaginatum* and between *P. notatum* × *P. indecorum*, all with diploid cytotypes. Additionally, Quarin (1983) obtained success in hybridizations between *P. notatum* ($2n = 40$) × *P. modestum* ($2n = 20$), demonstrating the cross viability of *Paspalum* with species combinations of differ ploidy levels. Within the same species, hybridizations in *P. notatum* have been successfully performed, both at the same ploidy level (Forbes & Burton, 1961; Silveira et al., 2022; Stein et al., 2004; Weiler et al., 2018) and between plants of different ploidy (Forbes & Burton, 1961; Martínez et al., 2007; Quarin et al., 1984). However, it is not clear the viability and the reproduction capacity of the adult plants obtained in that study. For *P. vaginatum*, different cultivars were released as the hybrids such as Sea Spray (United States patent number US7262341 B1; https://www.google.com/patents/US7262341), SeaIsle 1 (United States patent number US PP12,665 P2; https://patentimages.storage.googleapis.com/eb/49/9c/f1e0a825fa25d4/USPP12665.pdf), and Pure Dynasty (https://www.atlasturf.com/content/uploads/Atlas-Turf-Pure-Seed-Pure-Dynasty-Paspalum-brochure-1-21.pdf), among others (https://gapaspalum.com/seashore-paspalum-grasses/). For the other species addressed in this study, no reference to intraspecific crosses was found in the literature.

4.2 | The 22 *Paspalum notatum* accessions

The natural genetic variability and the possibility to obtain variability from crosses make feasible the intraspecific genetic improvement in *P. notatum*. The results demonstrate variability among the *P. notatum* accessions regarding the reproductive structures (sexual, obligate, and facultative apomictic) and the amount of DNA, which ranged from 1.7 to 3.54 pg. According to Blount et al. (2001) and Acuña et al. (2009), this species presents diploid and tetraploid materials, and most of the accessions evaluated in this work presented tetraploidy and apomixis.

Considering all the *Paspalum* spp. accessions, the *P. notatum* accessions were clustered in five of the six different groups by the Bayesian analysis (Figure 3). For a greater representativeness of genetic variability for this species, also represented by the great range of similarity coefficients, *K* value equal to four was chosen for *P. notatum* population structure (Figure 4) instead of the best *K* value equal to 2 (Figure S1). Although Espinoza et al. (2006) have found low genetic diversity among *P. notatum* accessions through the AFLP markers, our results showed large intraspecific genetic variability, corroborating with Cidade et al. (2008) and Reyno et al. (2012). Cidade et al. (2013) also found high genetic diversity among *P. notatum* accessions, with 15 accessions being common to the present study (BGPs 022, 026, 030, 034, 047, 155, 214, 216, 241, 242, 291, 294, 295, 304, and 306).

Regarding molecular analysis, the majority of accessions representing the Group IV were collected in southern Brazil and northern Argentina (Figure 4), which suggests an endemic distribution in these regions. The exception, BGP155 accession, collected in Macapá-AP at the north of Brazil, can be explained by possible transportation for landscape purposes, from the south to the north of Brazil by the migration of people during the north colonization that occurred decades before the collecting.

The reproduction mode characterization as completely sexual for BGP216 (Q 3664) differs from those obtained by Quarin et al. (1984) and Martínez et al. (2001), who already described it as facultative apomictic, presenting a high rate of sexuality (70%) and 15% of the ovules exclusively producing aposporic sacs. This genotype is an F_2 progeny resulting from the crossing of a sexual *P. notatum*, artificially tetraploidized (Forbes & Burton, 1961), with an apomictic strain called SWSB, which has white stigmas (Quarin, 1983; Quarin et al., 2003). However, the result obtained in this study can be related to the time of inflorescence collection, since, according to Quarin (1986), a sampling of embryo sacs with a higher rate of sexuality can be obtained at the end of the flowering season. The sexual reproduction mode was also detected for two diploid sexual accessions (BGP022 and BGP306).

The BGP241 *P. notatum* accession was classified with facultative apomictic reproductive structure, presenting 6% of embryonic sacs of the *Polygonum* type, 3% of embryonic sacs of the *Paspalum* type and 91% of sterile sacs. However, Zilli et al. (2015) reported that it is apomictic. Failures in the gametogenesis process can be related to the occurrence of embryo sacs sterility. Hosoo et al. (2005) reported that the occurrence of sterile embryo sacs in *Cryptomeria japonica* (L.f.) D. Don is due to failures in the meiotic process during the female gametes formation. Analyses regarding the meiotic behavior of the BGP241 accession must be carried out to confirm the reason for this occurrence.

The sexual accessions found in this study can be used as parents in crosses at ploidy level $2n = 4x$, either by chromosome duplication of a diploid genotype, or via direct use of an already tetraploid accession, as is the case of BGP216 (Q3664). The possibilities of performing crosses and selection based on different genetic backgrounds are real with the sexual accessions of *P. notatum* classified in the group III (accessions BGP216 and BGP306) and in the group IV (BGP022). Tetraploid individuals can be used directly in crosses with other apomictic individuals at this

ploidy level, allowing exploration of the existing variability between the different allelic pools, combining them in different possibilities.

The *P. notatum* genetic variability found in this work, regarding to the type of reproductive structure, ploidy level, or the divergence presented by the molecular markers, corroborate with the phenotypic variability of this species presented by Souza et al. (2012, 2020) and Castro et al. (2015). Among other accessions of different species, 17 accessions of *P. notatum* evaluated by Souza et al. (2012, 2020) are common with this study (BGPs: 022, 026, 030, 034, 115, 155, 214, 216, 241, 242, 291, 294, 295, 304, 306, 340, and 371) and considering the accessions evaluated by Castro et al. (2015), nine *P. notatum* accessions are common with this present study (BGPs: 026, 030, 115, 155, 214, 294, 295, 304, and 340). The cultivars of *P. notatum* var. *notatum* registered by EMBRAPA (cvs. Tuim [BGP026], Aruaí [BGP155], Tiriba [BGP295], and Maritaca [BGP318]) are apomictic tetraploids and each one has a different genetic background (Figure 4), thus being good candidates as male parents in crosses with sexual tetraploid material, including BGP216, which presents a percentage of all the four genic pools detected in the collection. These hybridizations will unlock the existing variability in the apomictic, leading to the obtaining of new cultivars with characteristics of interest.

5 | CONCLUSION

Characterizing *Paspalum* accessions by their reproductive systems, DNA content, chromosome number, and molecular diversity opens doors for controlled crosses between compatible parents. This unlocks the genus' genetic potential for creating (or enhancing) warm-season turfgrass cultivars. Equally crucial is the ongoing work of collecting, characterizing, and preserving new germplasm, thus valuing native flora.

AUTHOR CONTRIBUTIONS

Frederico de Pina Matta: Conceptualization; data curation; formal analysis; investigation; methodology; project administration; resources; software; supervision; validation; visualization; writing—original draft; writing—review and editing. **Alessandra Pereira Fávero**: Conceptualization; formal analysis; investigation; methodology; writing—review and editing. **Bianca Baccili Zanotto Vigna**: Conceptualization; data curation; formal analysis; investigation; methodology; software; writing—review and editing. **Marcelo Mattos Cavallari**: Conceptualization; writing—review and editing. **Fabio Alves**: Formal analysis; writing—review and editing. **Fernanda Ancelmo de Oliveira**: Formal analysis; investigation; methodology; writing—review and editing. **Anete Pereira de Souza**: Writing—review and editing. **Marisa Toniolo Pozzobon**: Formal analysis; investigation; writing—review and editing. **Ana Luisa Sousa Azevedo**: Formal analysis; methodology; writing—review and editing. **Marcos Rafael Gusmão**: Conceptualization; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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