

RAPD and ISSR markers in the evaluation of genetic divergence among accessions of elephant grass

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ABSTRACT. Considering the expected genetic variability of elephant grass (*Pennisetum purpureum*), due to its cultivation in different continents, we characterized and estimated the genetic divergences between 46 accessions of elephant grass with different edaphoclimatic adaptations, using RAPD and ISSR markers. We evaluated, comparatively, the consistency of the information achieved with these markers. Twenty-six RAPD and 25 ISSR primers were employed. The RAPD markers produced 185 bands, 72% of which were polymorphic, with a mean of 5.11 polymorphic bands per primer. The 25 ISSR starters produced 216 bands; 76% were polymorphic, with a mean of 6.56 polymorphic bands per primer. The correlation between the genetic distances achieved by the RAPD and ISSR markers was 0.76, which is highly significant by the Mantel test. Based on UPGMA grouping, considering the point of sudden change, five and six groups were formed for the data from the RAPD and ISSR markers, respectively.

These markers provided partially concordant groups, indicating that these techniques can provide consistent information and consequently could be used in studies of genetic diversity among accessions.

Key words: *Pennisetum purpureum* Schum.; RAPD and ISSR markers; Genetic variability

INTRODUCTION

Elephant grass (*Pennisetum purpureum* Schum.) comes from West Africa and is considered to be one of the major forage plants in the tropics and subtropics, due to its high yield, good forage quality and application versatility (Daher et al., 2002; Bhandari et al., 2006; Pereira et al., 2008; Harris et al., 2009; Struwig et al., 2009). Besides, this grass has potential to produce biofuels, such as charcoal, alcohol, and methane (Sanderson et al., 1996; Anderson et al., 2008; Strezov et al., 2008; Jakob et al., 2009; Morais et al., 2009; Lee et al., 2010).

This grass is an allotetraploid ($2n = 4x = 28$) of free pollination, and the large quantity of cultivars and genetic diversity result from its natural crossings (Bhandari et al., 2006). Therefore, the conservation of these materials in germplasm is very important for genetic breeding and to guard against unforeseen threats in agricultural production, such as epiphytes and climate change (Gepts, 2006; Gonçalves et al., 2008; Sudré et al., 2010).

In such context, active germplasm banks of elephant grass are maintained in many countries of the world, including South Africa, Brazil, Puerto Rico, the United States of America, Australia, China, Pakistan, and India (Bhandari et al., 2006). However, the accessions maintained in germplasm banks need characterization and evaluation, since, besides providing better knowledge about the germplasm available, which is essential for more intensive use in further stages, characterization and evaluation also allow the identification of duplicate accessions, settlement of nuclear collections and the identification of the modes of reproduction prevailing in the accessions, as well as of the occurrence or absence of intrinsic variability in individual accessions.

The characterization and evaluation may be based on agronomic, morphological, biochemical, molecular, and other information (Mohammadi and Prasanna, 2003; Gonçalves et al., 2009). However, molecular markers present more advantages because they reveal genetic differences with more details without the interference caused by environmental effects, providing fast results in the discrimination of the present diversity (Arif et al., 2010; Leal et al., 2010; Oliveira et al., 2010).

Some studies using molecular markers have been carried out in elephant grass aiming at achieving better understanding about diversity and kinship. RFLP (restriction fragment length polymorphism; Smith et al., 1993), RAPD (random amplified polymorphic DNA; Smith et al., 1993; Daher et al., 2002; Lowe et al., 2003; Passos et al., 2005; Pereira et al., 2008; Babu et al., 2009), AFLP (amplified fragment length polymorphism; Harris et al., 2009), and ISSR (inter-simple sequence repeats; Babu et al., 2009) are examples of such use.

The present study aimed at: i) characterizing and estimating the genetic diversity among 46 accessions of elephant grass from six countries, including forms which adapt to the edaphoclimatic conditions of Central and South America, using RAPD and ISSR markers, and ii) evaluating, comparatively, the congruence of the information achieved with the use of RAPD and ISSR markers.

MATERIAL AND METHODS

Plant material and DNA extraction

Forty-six clones of elephant grass (Table 1) were used. The total cell DNA was extracted from young leaves using a commercial kit (Plant Genomic DNA®). After the DNA extraction, DNA quantification was performed on agarose gels at 1.0%. The High DNA Mass Ladder (Invitrogen, USA) marker was used. The gel was stained with a mixture of 6X Blue Juice (0.4 mL 0.5 M 10X TAE; 0.2 mL 10% SDS; 0.2 mL bromophenol blue; 7.0 mL glycerol; 1.7 mL sterile water) with 5X GelRed (1 µL 10,000X GelRed in 0.5 mL dimethyl sulfoxide (DMSO); 2 mL ultrapure water), in a 1:1 proportion. The image was revealed using the resources of the MiniBis Pro photo-documentation system.

Table 1. Identification of the accessions, common name and provenance of 46 accessions of elephant grass from the UENF germplasm bank.

Identification	Common name	Provenance	Identification	Common name	Provenance
BGCE01	Merker S.E.A.	UFRRJ, km 47, Brazil	BGCE24	Costa Rica	Costa Rica
BGCE02	Merkeron Pinda	UFRRJ, km 47, Brazil	BGCE25	Híbrido Gigante da Colômbia	Colombia
BGCE03	Cubano Pinda	UFRRJ, km 47, Brazil	BGCE26	Elefante de Colômbia	Colombia
BGCE04	Merkeron Cubano de Pinda	Pindamonhangaba, SP, Brazil	BGCE27	Guaçu/IZ.2	Nova Odessa, SP, Brazil
BGCE05	Merkeron Comum	UFRRJ, km 47, Brazil	BGCE28	Napier Volta Grande	UFRRJ, km 47, Brazil
BGCE06	P-241-Piracicaba No. 9	Embrapa, CNPGL, Brazil	BGCE29	IJ 7141 cv EMPASC 306	Embrapa, CNPGL, Brazil
BGCE07	Elefante Híbrido 534-A	UFV, Brazil	BGCE30	Elefante de Pinda	Colombia
BGCE08	Mole de Volta Grande	Volta Grande, SP, Brazil	BGCE31	Merker 86, México	Colombia
BGCE09	Vrukwnona	Embrapa, CNPGL, Brazil	BGCE32	Três Rios	Nova Odessa, SP, Brazil
BGCE10	Napier	UFV, Brazil	BGCE33	Cameron, Piracicaba	UFRRJ, km 47, Brazil
BGCE11	Mineiro	Pedro Leopoldo, MG, Brazil	BGCE34	IAC, Campinas	UFRRJ, km 47, Brazil
BGCE12	Africano	UFRRJ, km 47, Brazil	BGCE35	Duro de Volta Grande Elefante	UFRRJ, km 47, Brazil
BGCE13	Taiwan A-148	UFRRJ, km 47, Brazil	BGCE36	Cachoeiro Itapemirim	UFRRJ, km 47, Brazil
BGCE14	Taiwan A-144	UFRRJ, km 47, Brazil	BGCE37	Gigante de Pinda	Pindamonhangaba, SP, Brazil
BGCE15	Capim - Cana D'África	EMCAPA, ES, Brazil	BGCE38	Porto Rico	UFRRJ, km 47, Brazil
BGCE16	King Grass	Cuba	BGCE39	Pusa Gigante Napier	India
BGCE17	Merker Santa Rita	UFRRJ, km 47, Brazil	BGCE40	Pusa Napier No. 1	India
BGCE18	Gramafante	RS, Brazil	BGCE41	Cuba-116	Cuba
BGCE19	Napier Goiano	GO, Brazil	BGCE42	Taiwan A-146	UFRRJ, km 47, Brazil
BGCE20	Merker	Limpa, MG, Brazil	BGCE43	Pasto Panamá	Panamá
BGCE21	Turrialba	UFRRJ, km 47, Brazil	BGCE44	Vrukwnona1	Embrapa, CNPGL, Brazil
BGCE22	Cameroon	Embrapa, CNPGL, Brazil	BGCE45	Albano	Colombia
BGCE23	Roxo	Lavras, MG, Brazil	BGCE46	Napier S.E.A.	UFRRJ, km 47, Brazil

RAPD and ISSR amplification

To achieve RAPD and ISSR fragments, first the selection of primers was performed and 26 and 25 primers were selected, respectively (Tables 2 and 3). The amplification reaction was carried out according to the protocol proposed by Williams et al. (1990), with some modification, for a final volume of 20 µL. The reaction contained the following concentration: 2 µL 10X buffer (500 mM KCl, 100 mM Tris-HCL, pH 8.4), 2 µL 25 mM MgCl₂, 1.6 µL 2 mM dNTPs, 1 µL DMSO, 1.8 µL 0.5 mM primer, 0.12 µL 5 U Taq DNA polymerase and 2 µL 5 ng genomic DNA. The final volume was completed with ultrapure water. The polymerase chain reactions (PCR) for the RAPD marker were conducted as follows: 4 min at 94°C, continuing for 45 cycles (94°C for 1 min, 35°C for 1 min and 72°C for 3 min), and a final extension at 72°C for 7 min. In relation to the ISSR markers, the PCRs followed this routine: 3 min at 94°C,

continuing for 42 cycles [94°C for 1 min, 30-57°C for 1 min (depending on the starter used) and 72°C for 3 min], and a final extension at 72°C for 7 min.

The products of the amplifications were separated on 1.5% agarose gel. The 1-kb DNA Ladder (Invitrogen) marker was used. The gel was stained with a mixture of 6X Blue Juice (0.4 mL 0.5 M 10X TAE; 0.2 mL 10% SDS; 0.2 mL bromophenol blue; 7.0 mL glycerol; 1.7 mL sterile water) with 5X GelRed (1 µL 10,000X GelRed in 0.5 mL DMSO; 2 mL ultra-pure water), at a 1:1 proportion. The image was revealed by the resources of the MiniBis Pro photo-documentation system.

Data analysis

For the analysis of RAPD and ISSR markers, the gels revealed were visualized and, later, interpreted by the presence or absence of bands, with the generation of a binary matrix. To estimate the genetic distances between genotypes, the Jaccard similarity coefficient was used. Later, the Pearson correlation was carried out, with the use of the Mantel test (10,000 permutations), among the distance matrices of the RAPD and ISSR markers. The simplified representation of the genetic distances between the accessions was achieved by the UPGMA (unweighted pair-group method using arithmetic average) method and represented by a dendrogram. All the analyses were carried out using the R system software (<http://www.r-project.org>).

RESULTS AND DISCUSSION

RAPD marker

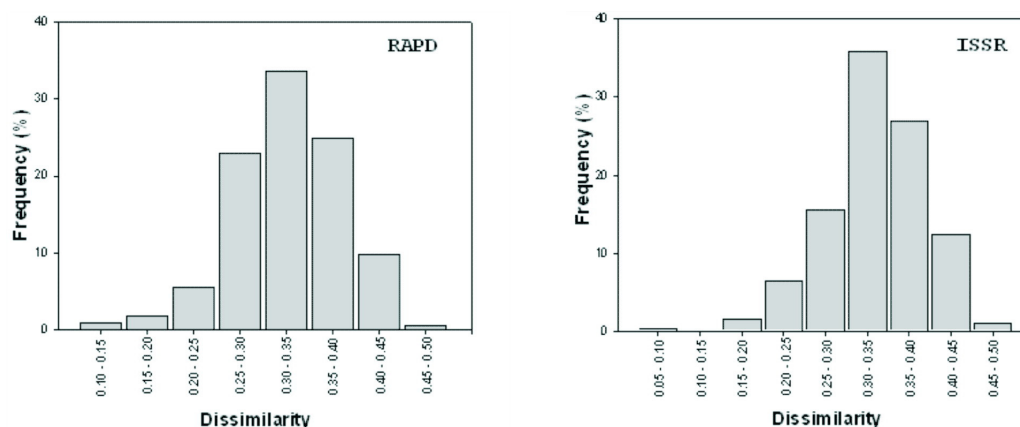
By RAPD analysis, each primer produced easily detectable bands with variable intensity, and nonspecific bands, which were discarded. The 26 primers used produced 185 bands (Table 2). Of these, 133 were polymorphic (71.89%) and 52, monomorphic (28.11%). The number of polymorphic bands varied from 2 to 8, which occurred with the OPC-04 and OPAA-20 primers, respectively. The level of polymorphism verified in the present study is in accordance with those achieved by Pereira et al. (2008) and Passos et al. (2005), but lower than those achieved by Babu et al. (2009), who evaluated 30 accessions of elephant grass and detected 87.84% polymorphism.

The analysis of the frequency distribution of the dissimilarity of 1035 combinations for the pairs of the 46 accessions of elephant grass revealed that the distribution was uniform, ranging from 0.10 to 0.47 with an average of 0.33 (± 0.04), which is higher than that achieved by Pereira et al. (2008) and Passos et al. (2005), who verified an average distance of 0.21 and 0.25, respectively (Figure 1). The classes 0.30-0.35 and 0.35-0.40 revealed the highest frequencies, with magnitudes of 33.62 and 24.83, respectively. It is possible, therefore, to infer that RAPD markers were efficient in detecting the genetic variability among the accessions evaluated in the present study. The accessions BGCE09 (Vruckwona) and BGCE30 (Elefante de Pinda) were the most genetically distant, with the value of 0.47, while BGCE34 (IAC-Campinas) and BGCE44 (Vruckwona1) were the closest (0.11).

Five groups were formed by a cut at the distance of 0.31, considering the most abrupt turning point in the dendrogram (Figure 2). Groups I and II gathered 71.74% of the accessions.

Table 2. Number of polymorphic and monomorphic bands obtained with the use of RAPD markers in 46 accessions of elephant grass, using 26 primers.

Primers	Bands		Total
	Monomorphic	Polymorphic	
OPA-2	4	5	9
OPA-8	1	4	5
OPA-9	2	6	8
OPAA-11	3	6	9
OPAA-16	3	3	6
OPAA-20	2	8	10
OPAB-2	2	5	7
OPAB-4	1	6	7
OPAB-6	2	6	8
OPAB-9	1	6	7
OPAC-12	1	7	8
OPAC-17	2	6	8
OPAD-11	1	8	9
OPAE-11	2	8	10
OPAE-18	3	3	6
OPAW-15	1	5	6
OPAW-9	2	3	5
OPB-17	2	3	5
OPC-11	1	7	8
OPC-12	2	3	5
OPC-16	3	3	6
OPC-4	4	2	6
OPD-13	2	4	6
OPD-20	1	6	7
OPN-15	2	4	6
OPN-5	2	6	8
Total	52	133	185

**Figure 1.** Frequency distribution of the dissimilarity based on RAPD and ISSR markers among the 46 accessions of elephant grass.

Group I comprised 17 accessions, and Group II, 16 accessions. Groups III, IV and V were formed by 1, 2 and 10 accessions, respectively (Figure 2). The accessions BGCE9, BGCE34 and BGCE15 (Vruckwona, IAC-Campinas and Capim Cana d' África, respectively) allocated in Group II were also allocated in the same group in the results of Passos et al. (2005) and Pereira et al. (2008). The accessions BGCE10 and BGCE11 (Napier and Mineiro, respec-

tively) were gathered in Group I, which also occurred with the results achieved by Daher et al. (1997a) when using enzyme standards; by Daher et al. (1997b) and Shimoya et al. (2002), using morphoagronomic descriptors, and by Passos et al. (2005) and Pereira et al. (2008).

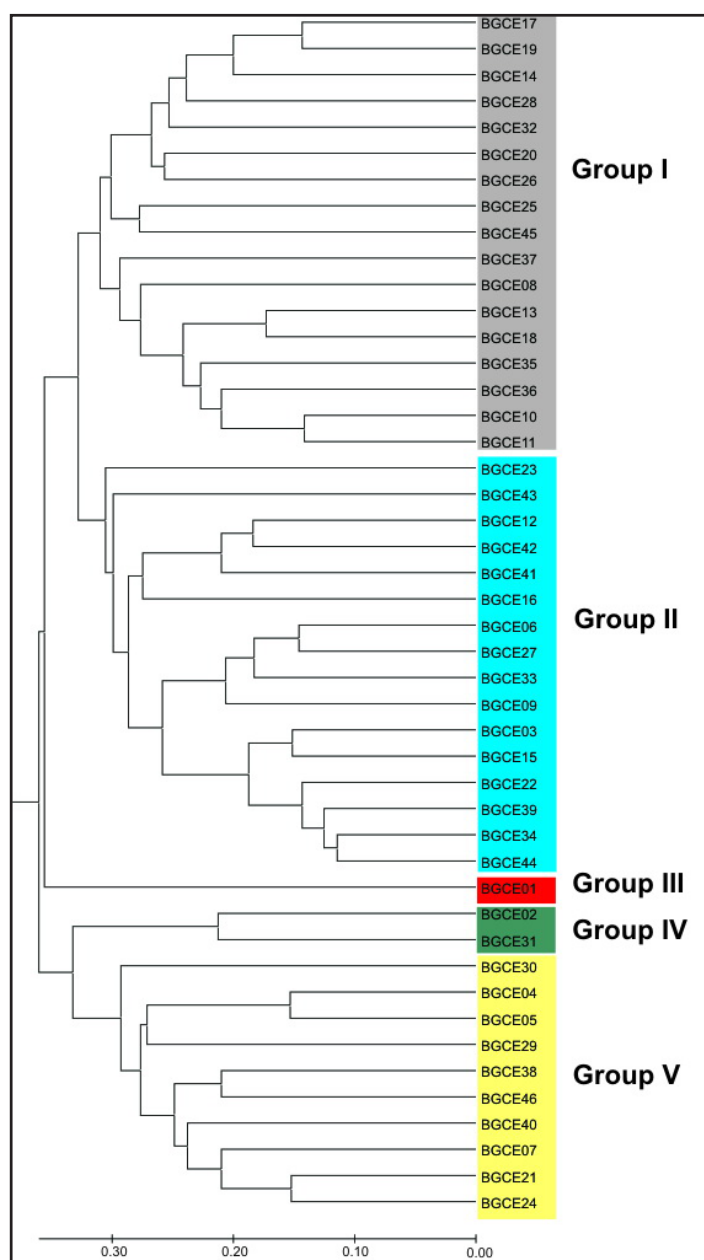


Figure 2. Dendrogram of genetic divergence among 46 accessions of elephant grass using the Jaccard coefficient based on the UPGMA algorithm from RAPD data.

ISSR marker

From the analysis of the ISSR markers, the 25 primers used produced 216 bands clearly visualized on the gels. Of these, 164 were polymorphic (75.93%) and 52, monomorphic (24.07%) (Table 3). The starter (AG)₈CTA was the most informative, providing the amplification of 11 fragments, while (GT)₆CC presented the smallest quantity of polymorphic fragments, a total of only two.

Table 3. Number of polymorphic and monomorphic bands obtained with the use of ISSR markers in 46 accessions of elephant grass, using 25 primers.

Primers	Bands		Total
	Monomorphic	Polymorphic	
(GAGA) ₃ CC	1	7	8
(GT) ₆ CC	3	2	5
(AC) ₃ GC	3	6	9
(AC) ₃ CT	2	5	7
(AC) ₃ YG	4	6	10
(GGAT) ₃ GA	4	4	8
(GAA) ₄ AA	0	9	9
(CT) ₃ G	1	8	9
(AC) ₃ T	2	8	10
(AG) ₃ YT	1	8	9
(AG) ₃ YA	4	5	9
(ATG) ₆	0	6	6
(GACA) ₄	0	9	9
DBD(AC) ₇	3	5	8
(GA) ₃ C	2	7	9
(GA) ₃ T	5	6	11
(AC) ₃ C	2	7	9
(ACT) ₆ T	2	6	8
(ACT) ₆ C	2	8	10
(ATG) ₆ G	2	5	7
G(CTA) ₆	2	4	6
(GA) ₃ T	0	10	10
(GA) ₃ AC	2	7	9
(ACC) ₄ Y	5	5	10
(AG) ₈ CTA	0	11	11
Total	52	164	216

The analysis of the frequency distribution of the dissimilarity revealed uniform distribution, ranging from 0.05 to 0.50, with an average of 0.34 (± 0.05), and the classes 0.30-0.35 and 0.35-0.40 presented the highest frequency, with estimates of 35.84 and 26.96, respectively (Figure 1). These results agree with those achieved with the RAPD markers, indicating that both markers were efficient in detecting the genetic similarity and dissimilarity among the accessions evaluated in the present study. BGCE12 (Africano) and BGCE30 (Elefante de Pinda) presented the highest values for the genetic distance (0.48) for the ISSR marker. The same accessions presented a genetic distance of 0.38 for the RAPD technique, while BGCE09 and BGCE30, the most divergent for these markers, revealed a genetic distance of 0.43 by the ISSR markers, corroborating the high genetic separation among these accessions. The accessions BGCE04 (Merkeron Cubano de Pinda) and BGCE05 (Merkeron Comum) were the most similar, with a genetic distance of 0.06. They presented a distance of 0.15 by the RAPD marker, while BGCE34 and BGCE44, the most similar by the RAPD technique, revealed a genetic distance of 0.17, corroborating the genetic proximity among them.

Six groups were formed based on a cut performed at a distance of 0.31, consider-

ing the most abrupt point of change in the dendrogram (Figure 3). Groups I and VI gathered 80.43% of the accessions; Group I comprised 18 accessions, and Group VI, 19 accessions. Groups II, III, IV, and V were formed by 6, 1, 1, and 1 accessions, respectively (Figure 3). The union of the accessions BGCE09, BGCE34 and BGCE15 in the same group (Group VI), and BGCE10 and BGCE11, in Group I, corroborates the results achieved by the RAPD marker, which indicates the validity of the use of both techniques for the accessions studied.

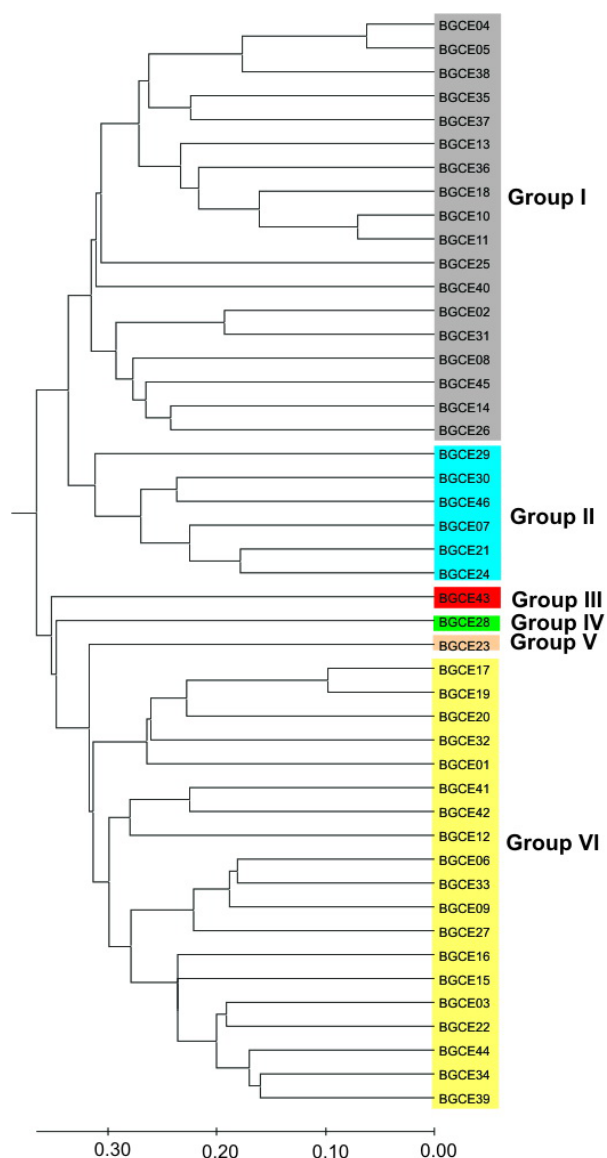


Figure 3. Dendrogram of genetic divergence among 46 accessions of elephant grass using the Jaccard coefficient based on the UPGMA algorithm from ISSR data.

Comparison between the RAPD and ISSR markers

The estimate of the correlation between the genetic distances achieved by the RAPD and ISSR markers was 0.76, with probability $P < 0.001$ by the Mantel test (10,000 permutations), indicating that there is a pattern of association between the results achieved by these two analytical procedures in the discrimination of the accessions. Concordant results between RAPD and ISSR have been observed in other articles (Behera et al., 2008; Muthusamy et al., 2008; Arif et al., 2009).

Babu et al. (2009), evaluating the variability among 30 accessions of elephant grass using RAPD and ISSR, verified low correlation ($r = 0.33$) between the markers, indicating low correspondence between the polymorphisms brought by these techniques. However, when genotypes were evaluated by the UPGMA grouping method, the authors verified that both techniques were efficient in the geographical identification of the accessions.

The UPGMA grouping analysis allowed us to observe the association between the two markers evaluated here. Of the 17 accessions of Group I, gathered based on the RAPD markers, 12 were allocated in Group I (BGCE08, BGCE10, BGCE11, BGCE13, BGCE14, BGCE18, BGCE25, BGCE26, BGCE35, BGCE36, BGCE37, and BGCE45) and 4, in Group VI (BGCE17, BGCE19, BGCE20, and BGCE32) of the ISSR markers. Besides, in Group II of the RAPD marker, formed by 16 accessions, 14 accessions were allocated in Group VI (BGCE03, BGCE06, BGCE09, BGCE12, BGCE15, BGCE16, BGCE22, BGCE27, BGCE33, BGCE34, BGCE39, BGCE41, BGCE42, and BGCE44), based on the ISSR markers. This indicates that both techniques can provide consistent information for diversity analyses in accessions of elephant grass from different origins, with different edaphoclimatic adaptations.

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