



High degree of genetic diversity among genotypes of the forage grass *Brachiaria ruziziensis* (Poaceae) detected with ISSR markers

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ABSTRACT. The grasses of the genus *Brachiaria* account for 80% of the cultivated pastures in Brazil. Despite its importance for livestock production, little information is available for breeding purposes. Embrapa has a population of *B. ruziziensis* from different regions of Brazil, representing most of existing variability. This population was used to initiate an improvement program based on recurrent selection. In order to assist the genetic improvement program, we estimated the molecular variability among 93 genotypes of Embrapa's collection using ISSR (inter-simple sequence repeat) markers. DNA was extracted from the leaves. Twelve ISSR primers generated 89 polymorphic bands in the 93 genotypes. The number of bands identified by each primer ranged from two to 13, with a mean of 7.41. Cluster analysis revealed a clearly distinct group, containing most of the *B. ruziziensis* genotypes apart from the outgroup genotypes. Genetic similarity coefficients ranged from 0.0 to 0.95, with a mean of 0.50 and analysis of molecular variance indicated higher variation within (73.43%) than among species (26.57%). We conclude that there is a high genetic diversity among these *B. ruziziensis* genotypes, which could be explored by breeding programs.

Key words: Genetic variability; Forage; Molecular markers

INTRODUCTION

Pastures are very important for agriculture in Brazil since they account for 75% of the agricultural lands in the country. The main feeding source of the enormous cattle herd in Brazil (200 million head) is through forage. Pastures, as sole food source, are responsible for almost 90% of beef and the majority of 25.7 billion liters of milk produced annually in the country (Souza Sobrinho et al., 2009). Starting in 1960, mainly during the 1970s and 1980s, areas of natural grassland have been replaced by cultivated pastures, mainly with species of the genus *Brachiaria* (Martha Júnior and Corsi, 2001). Of a total of 180 million hectares covered by pastures in the country, it is estimated that cultivated pastures account for 105 million hectares and 80% of these are cultivated with *Brachiaria* grasses (Pereira, 1998; Dusi, 2001).

In Brazil, the most cultivated species of *Brachiaria* are *B. decumbens*, *B. brizantha*, *B. humidicola* and *B. ruziziensis*. Among these species, *B. ruziziensis* is the only sexual and diploid species, allowing the crossing of genotypes and generation of variability for selection. Although *B. ruziziensis* is not a species with a large cultivated area, the demand for seeds is increasing following the advancement of integration between agriculture and livestock production.

Integration between agriculture and livestock production is an important alternative to pasture recuperation and improvement of annual crops. It increases straw to minimum tillage systems, improves soil chemical, physical and biological properties, allows better use of equipment, and improves farmers' income and jobs in rural areas (Macedo, 2009). *B. ruziziensis* has been commonly used in agriculture livestock integrated systems mainly due to its better adaptation to overseeding, less herbicide demand for drying and establishment of the next crop, and also its small tussock architecture.

Despite the importance of *Brachiaria* for livestock production, the available information about the biology and genetics of this genus is very limited, especially for breeding purposes. Introduction of genetic variability in cultivated species was somehow accomplished with few crossings and introduction of different accessions. Natural recombination may have also occurred during the past 50 years that *Brachiaria* was introduced in Brazil. Characterization of genetic diversity in a population is required for better exploitation of genetic resources in breeding programs, and is a critical factor that contributes to preserve biodiversity. Knowledge of genetic diversity is essential in the development of commercial hybrids. Diversity can be assessed using many tools including DNA markers (Kapila et al., 2008).

Molecular markers are valuable tools in the characterization and evaluation of genetic diversity within and between species and populations. Knowing the degree of genetic similarity among different genotypes is useful to organize a working collection, to identify heterotic groups, and to select genotypes for crosses (Bonato et al., 2006). It has been shown that different markers may reveal different classes of variation (Powell et al., 1996; Russell et al., 1997). This could be explained by the genome fraction surveyed by each type of marker, their distribution throughout the genome and the extent of the DNA target which is analyzed by each specific assay (Dávila et al., 1999). Molecular markers such as random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR), simple sequence repeats (SSR) and amplified fragment length polymorphism (AFLP) have been successfully used to assess the genetic diversity in cultivars of many plant species (Balasaravanan et al., 2003; Khan et al., 2005; Martos et al., 2005; Terzopoulos et al., 2005). ISSR markers show some advantages since these markers overcome the shortcomings of the low reproducibility of RAPD, the high cost of AFLP and the complex generation of SSR

(Reddy et al., 2002), and represents a fast and cost-efficient technique.

Selection and assessment of genotypes with ISSR markers could aid breeding processes by separating closely related genotypes, thus increasing the efficiency and orientation of future crossings and genetic studies. The Brazilian Agricultural Research Corporation (Embrapa) coordinates the genetic improvement program of *B. ruziziensis*. This program aims to identify high-yield genotypes with improved forage quality and resistant to major biotic and abiotic stresses. In order to assist the *B. ruziziensis* improvement program, this study aimed to estimate the genetic variability among *B. ruziziensis* genotypes, and contribute with information about the genetic basis of Embrapa's collection through the use of ISSR markers. The results of this work will be used for monitoring the genetic variability of genotypes under evaluation and also to direct crosses between divergent genotypes to explore heterosis. The information obtained will be important for defining breeding strategies and methods to be adopted by the program in order to make it more dynamic, increasing the chances of successful selection.

MATERIAL AND METHODS

A total of 93 genotypes of Embrapa's collection of *B. ruziziensis* genetic improvement program were selected for this diversity study. Outgroup genotypes were used to provide a reference point against which genetic similarity of *B. ruziziensis* could be evaluated for generating the trees. The genotypes used as outgroup were: *B. brizantha* (cvs. Marandu and Xaraes), *B. decumbens* (cv. Basilisk), *B. humidicola* (cv. Llanero), *Brachiaria* spp (*B. spp*) and millet (*Pennisetum glaucum*).

Leaves were harvested and genomic DNA was extracted based on the protocol described by Ferreira and Grattapaglia (1995) with modifications. ISSR reactions contained: 0.5 mM primer, 100 mM Tris-HCl (pH 8.4), 500 mM KCl, 1.5 or 2.0 mM MgCl₂ (depending on the primer), 0.15 mM dNTP, 1 U Taq DNA polymerase and 30 ng genomic DNA in a final volume of 25 µL. The 12 primers used were developed at the University of British Columbia Laboratory of Biotechnology (UBC-808, UBC-809, UBC-815, UBC-820, UBC-823, UBC-826, UBC-830, UBC-841, UBC-845, UBC-851, UBC-857, UBC-859). The amplifications were performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation at 94°C for 4 min, followed by 45 cycles of one minute at 94°C, 45 s at 50°C and 2 min at 72°C, and a final extension step of 7 min at 72°C.

The DNA fragments were separated by electrophoresis on 2% agarose gels, for 5 h at 120 V. Each gel was stained by immersion in 3 mg/mL ethidium bromide solution for 30 min and then photographed under ultraviolet light using the Eagle Eye II photo documentation system (Stratagene, La Jolla, CA, USA). The size of amplified fragments was estimated by comparison with a 200-bp molecular ladder (Promega, Madison, WI, USA).

The scoring of the markers was performed using the RFLPscan program (Scanalytics, Fairfax, VA, USA). Only fragments of good resolution and high intensity were considered in the analysis. The allelic data for all genotypes was scored in the form of a binary matrix, where 1 represented the presence and 0 the absence of a DNA band. The genetic distance for all the pairwise combinations was calculated using Jaccard's similarity coefficient (Jaccard, 1908). The genetic distance matrix was used to generate a dendrogram using the unweighted pair group method with arithmetic mean (UPGMA). The consistency of the dendrogram was eval-

uated by the correlation between the dissimilarity matrix and cophenetic matrix (Sokal and Rohlf, 1962). The analysis of molecular variance (AMOVA) to check population structuring was carried out according to Excoffier et al. (1992), using the software GENES (Cruz, 2008).

RESULTS AND DISCUSSION

The 12 ISSR primers generated 89 polymorphic bands, which were used to estimate the genetic diversity among 93 genotypes of *B. ruziziensis* and the six outgroup genotypes. The number of bands identified in each primer ranged from two (primer UBC-826) to 13 (primer UBC-823) with an average of 7.41 bands per primer (Table 1). Monomorphic bands were not identified, which could be an indication of the high variability among the genotypes. The length of the bands ranged from 300-1560 bp. A total of 33 unique bands were identified in all genotypes: 15 bands were found in *B. ruziziensis* (genotypes 7, 8, 11, 27, 42, 57, 58, 97, 101 and 102) and 18 bands belonged to the outgroup. Similar results were obtained by Ambiel et al. (2010), who conducted a study with 10 RAPD markers and 42 samples, including different species of *Brachiaria*. They found 114 bands (all polymorphic) with an average of 11.4 bands/primer. Guaberto (2009) identified 184 polymorphic bands in a total of 202 (14 bands/primer), using 14 RAPD markers in *B. ruziziensis*.

Table 1. Number of bands generated for each ISSR primer used to amplify the DNA of 93 genotypes of *Brachiaria ruziziensis* and six outgroup genotypes.

Primer	Number of bands	Primer	Number of bands
UBC-808	12	UBC-830	7
UBC-809	6	UBC-841	11
UBC-815	9	UBC-845	3
UBC-820	12	UBC-851	7
UBC-823	13	UBC-857	3
UBC-826	2	UBC-859	4

In general, ISSR markers detect high levels of polymorphism (Powell et al., 1996; Joshi and Dhawan, 2007; Sreedhar et al., 2007). ISSR fingerprinting detected more polymorphic bands (12.8%) than RAPD fingerprinting (4.28%) in micropropagated tea clones (Devarumath et al., 2002). Prevost and Wilkinson (1999) used only four ISSR primers to completely distinguish 34 potato cultivars. In our study, 89 bands (all polymorphic) generated by 12 ISSR primers were able to explore the genomic variation among 93 genotypes of *B. ruziziensis*.

Cluster analysis revealed by the dendrogram indicates genetic variability among the *B. ruziziensis* genotypes (Figure 1). There is a clear formation of a group containing most of the genotypes of *B. ruziziensis* distinct from the outgroup genotypes. Besides, three genotypes of *B. ruziziensis* were gathered with the outgroup (42, 74 and 68). The UPGMA cluster analysis clustered the 99 genotypes into three main groups (Figure 1). Group A included 92 genotypes of *B. ruziziensis* and four outgroup genotypes [*B. decumbens*, *B. brizantha* (cv. Marandu and Xaraes) and *B. spp.*]. Group B consisted of only one genotype of *B. ruziziensis* (genotype 42), and group C included two outgroup genotypes (*B. humidicola* and *Pennisetum glaucum*). The dendrogram showed a cophenetic correlation matrix of 0.87, indicating a high goodness-of-fit. Similar results were obtained by Ambiel et al. (2010), assessing the genetic divergence among accessions from different *Brachiaria* species by RAPD markers, where *B.*

humidicola was separated from all the other *Brachiaria* species and where *B. ruziziensis*, *B. decumbens* and *B. brizantha* were clustered in the same group by the dendrogram generated from genetic dissimilarity estimates.

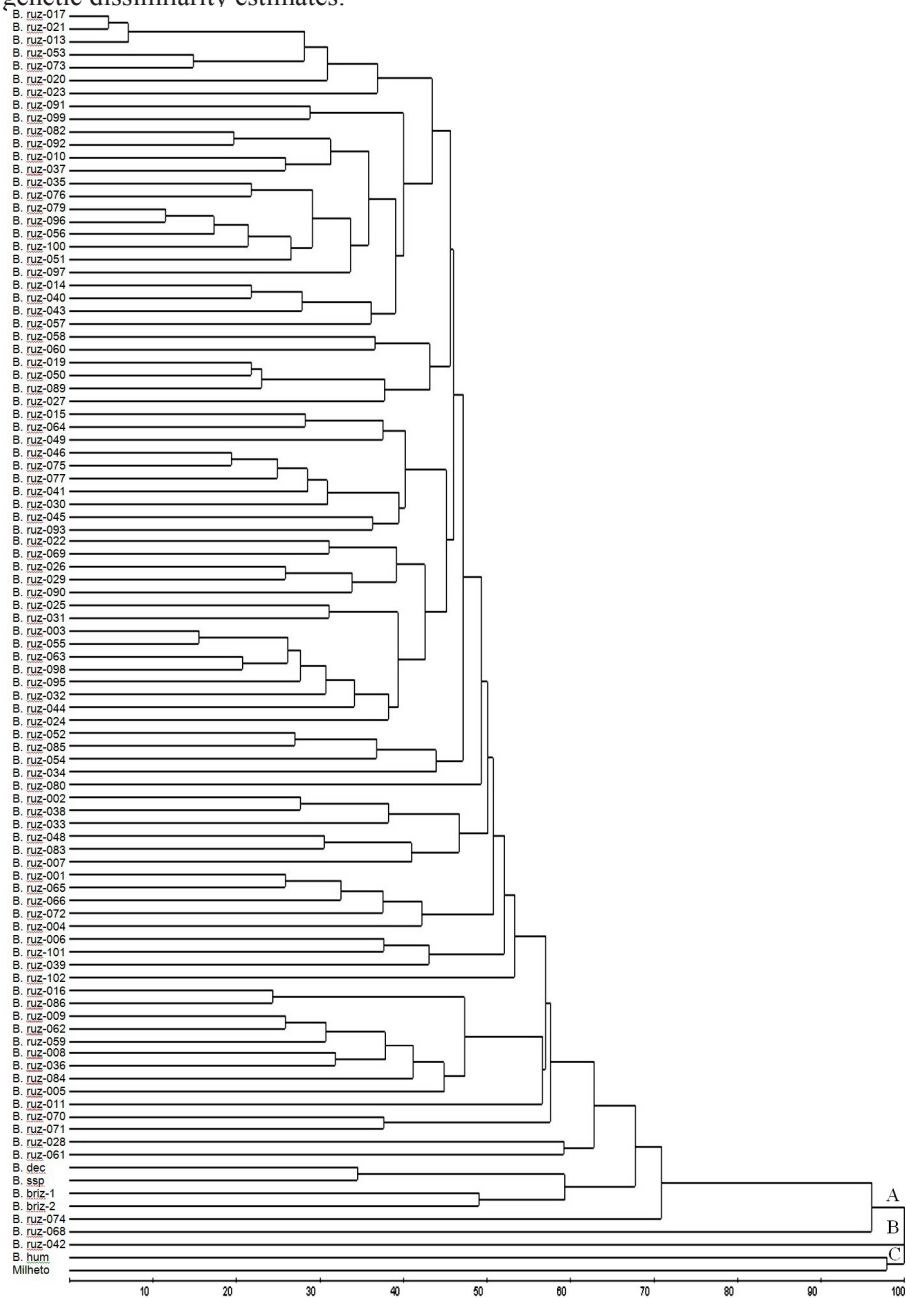


Figure 1. Dendrogram analysis of 93 *Brachiaria ruziziensis* and six outgroup genotypes revealed by ISSR markers, generated by Jaccard's similarity coefficient.

A similarity matrix for the 99 genotypes was constructed. The frequency distribution of the coefficients indicated an average coefficient between genotypes of 0.47 (Figure 2). Between *B. ruziziensis* genotypes only, the genetic similarity coefficients ranged from 0 to 0.95, with an average of 0.50, attesting to the genetic variability within the species. We also found that 84% of the estimated coefficients showed values below 0.6, and 99 pairs did not share any bands, reflecting the high level of genetic variability among the genotypes used in this study. Souza Sobrinho et al. (2009 and 2010) also reported the existence of high genetic variability in *B. ruziziensis* for traits related to yield and quality of forage produced as well as for tolerance to biotic and abiotic stresses. Chiari et al. (2008) used RAPD markers to assess the genetic variability of four species of *Brachiaria* and concluded that the greatest dissimilarities were observed among *B. ruziziensis* genotypes (0.73 to 0.83).

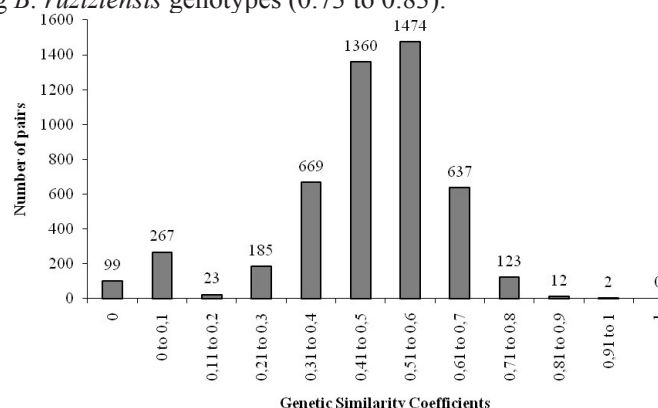


Figure 2. Frequency distribution of genetic similarity coefficients between 93 *Brachiaria ruziziensis* and six outgroup genotypes.

Among the genotypes, a total of 89 polymorphic bands were identified. Thirty three were found to be unique bands from 10 genotypes of *B. ruziziensis* (7, 8, 11, 27, 42, 57, 58, 97, 101 and 102), and the other bands were identified in the outgroup. Genotype 42 showed six unique bands, which highlights its highest divergence when compared to all other genotypes. These unique bands could be used to quickly and reliably identify this genotype, which could be extremely useful in the breeding program. Confirmation of hybridization after execution of controlled crossings, for instance, is a practical example of the usefulness of these markers. Within the genus *Brachiaria*, such crossings are usually carried out without emasculating the flowers and the identification of hybrid individuals is highly demanded by the breeding programs. Genetic relationship based on molecular markers is helpful in selecting parental combinations for hybrid crop breeding so as to gain desirable traits (Becelaere et al., 2005; Ali et al., 2008). These results could be used as auxiliary tools to help *Brachiaria* breeders to select appropriate parents in their breeding programs to maximize yield as well as to maintain genetic diversity. The use of genotypes of high genetic diversity is a strategy used by breeders to maximize heterosis or hybrid vigor. Thus, the recommendation of crossings should prioritize the use of genotype or group of genotypes with high genetic distances.

The UPGMA dendrogram shows high genetic variability among *B. ruziziensis* geno-

types (Figure 1). These conclusions were further strengthened by AMOVA, which indicated more variation within (73.43%) than between populations (26.57%) (Table 2). Cross-pollinating species are, by definition, much more variable than self-pollinating species (Yanaka et al., 2005). *B. ruziziensis* is a cross-pollinating species (Souza Sobrinho et al., 2009), so it is expected that greater variability was found within the species. Guaberto (2009) and Ambiel et al. (2010) worked with RAPD markers in different species of *Brachiaria* and found similar results, which confirms the predominance of genetic variability within species. Higher variation within groups was also observed in cross-pollinating species such as Buffalo grass (Huff et al., 1993), *Lolium multiflorum* (Vieira et al., 2004), *Bromus innermis* (Diaby and Casler, 2005); *Bromus riparius* Rehm (Ferdinandez et al., 2001), *B. auleticus* (Rivas, 2001), *Chloris gayana* K. (Ubi et al., 2003), *Pascopyrum smithii* (Larson et al., 2003) and *Eucalyptus globulus* (Nesbitt et al., 1995). In Napier grass, RAPD analysis found 35% variation between and 65% within groups based on geographical origin (Lowe et al., 2003). Thus, the present material indicates wide range of diversity available for breeding. This large variability reduces the chance of genetic drift and inbreeding, thus contributing to the maintenance of high genetic diversity within populations.

Table 2. Analysis of molecular variance between and within *Brachiaria* populations.

Source of variation	d.f.	Sum of squares	Mean squares	% variation
Between populations [#]	1	28.281	28.28	26.57
Within populations	97	540.10	5.56	73.43
Total				

[#]Population 1: *Brachiaria ruziziensis*; Population 2: outgroup genotypes.

According to Hamrick (1983), the distribution of genetic variation is not random within populations; it is determined by the reproductive system, geographic distribution, effective size of populations, mode of reproduction and gene flow through dispersal of pollen. Besides these features, evolutionary factors acting on the genetic diversity, introducing new alleles or modifying the frequencies (Weir, 1990). The existence of genetic variability within *B. ruziziensis*, revealed by molecular markers, increases the chance of success of the *B. ruziziensis* breeding program, since this is the only species of *Brachiaria* grown in Brazil that is diploid and sexual, allowing recombination and selection of superior genotypes (Souza Sobrinho et al., 2009). Furthermore, the molecular characterization of genotypes is also important for monitoring the genetic variability along the selection cycles. Thus, the estimates of genetic divergence, based on molecular markers, will help to determine if the rate of selection is appropriate, as well as to maintain sufficient variability to select the breeding cycles.

CONCLUSION

ISSR markers were effective in assessing the genetic variability within genotypes of *Brachiaria ruziziensis*, given the large number of markers identified and the high polymorphism found, and the data suggest that this population has a wide genetic base suitable for breeding.

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