academicJournals

Vol. 16(12), pp. 607-614, 22 March, 2017
DOI: 10.5897/AJB2016.15638
Article Number: D0C7EE863226
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Differentiation of *Urochloa brizantha* cultivars by inter-simple sequence repeat (ISSR) markers in seed samples

Inaê Braga[1], Claudia Jaqueline Tome Yamamoto[2], Ceci Castilho Custódio[2] and Nelson Barbosa Machado-Neto[2]*

[1]Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), Instituto de Biociências, Biologia Vegetal, Rio Claro-SP, CEP: 13506-900, Brazil.
[2]Agronomy Program, Universidade do Oeste Paulista (UNOESTE), Presidente Prudente, SP, CEP19067175, Brazil.

Received 31 August, 2016; Accepted 10 February, 2017

Approximately 80-90% of cultivated grassland areas in Brazil are covered by *Urochloa brizantha* [syn. *Brachiaria brizantha* (Hochst. ex A. Rich.) Stapf.]. Some genotypes of *Urochloa* have being widely used with a wrong nomenclature, like species and cultivars. In this way, the *Urochloa* cultivar identification is primordial for breeding programs and seed production. Considering the importance of genetic purity in commercialized seed lots, the present work aimed to evaluate the use of inter-simple sequence repeat (ISSR) markers in six cultivars of *U. brizantha* (Xaraés; Piatã; Basilisk; MG4; MG5 and Marandu) to discriminate and determine the contamination in seed batches. Results showed that it is possible to discriminate all cultivar with only two primers in pure samples. Basilisk was confirmed as a *U. brizantha* cultivar. ISSR markers showed a low polymorphism level. It was not possible to separate samples intentionally contaminated even at 5%.

Key words: *Brachiaria*, molecular markers, varietal purity.

INTRODUCTION

The *Urochloa* genus (syn. *Brachiaria*) belongs to the Poaceae family and was introduced in Brazil from Africa, having as its main centre of origin and diversification, the east of the continent (Vilela, 2005). In Brazil, sixteen species were introduced from Africa and the most important were *Urochloa brizantha*, *Urochloa decumbens*, *Urochloa ruziziensis* and *Urochloa humidicola* (Karia et al., 2006.), which represent the majority of commercial seeds. In addition to its use as pasture, forage species have been used to cover the soil in crop-livestock integration (Crusciol et al., 2009). Currently, it is estimated that more than 50% of the area cultivated with pastures in the Center-west region of the country are cropped with this grass (Macedo, 2006).

*Corresponding author. E-mail: ceci@unoeste.br or nbmneto@unoeste.br.

There are seven major worldwide collections of *Urochloa*, all *ex situ*, which have a total of 987 accesses of 33 described species (Keller-Grein et al., 1996). However, problems with incorrect identifications are still frequent among *Urochloa* species, as well as in the accessions of germplasm collections (Assis et al., 2003). Presently, Embrapa Gado de Corte is responsible for the largest and most important resources of Forage Germplasm Bank of the *Urochloa* used for the plant breeding efforts (Valle et al., 2008).

Currently, nine cultivars of *U. brizantha* are registered, but just Marandu; Xaraés; BRS Piatã; BRS Paiaguás; Basilisk; MG4; MG5 Vitória and MG-13 Braúna are commercially available (Brasil, 2016), and there are still doubts about the identification of some of these cultivars. An important cultivar, originally introduced as *Urochloa decumbens*, is Basilisk, collected originally in Uganda, sent to Australia and subsequently introduced from International Research Institute (IRI) to São Paulo in the early 1960s and was the first *Urochloa* cultivar (Karia et al., 2006). According to Renvoize et al. (1996), Basilisk was wrongly identified as a *U. decumbens* but it is actually an *U. brizantha*, data is supported by Ambiel et al. (2008, 2010).

The Marandu cultivar was introduced in 1967 in São Paulo, and occupies the largest grassland area in Brazil (Macedo, 2006). More recently, the cultivars Xaraés, BRS Piatã and BRS Paiaguás were released by Embrapa Gado de Corte (CNPGC) (Brasil, 2016).

The cultivars, MG-4, MG-5 'Vitória' and MG-13 'Braúna' were released by Matsuda company after an accession selection from CIAT genebank (International Center for Tropical Agriculture) (Matsuda, 2016). Despite some data pointing that Xaraés and MG-5 are not the same material (Ambiel et al., 2008, 2010), for the National Cultivar Register, they are under the same number (Brasil, 2016).

Assis et al. (2003) succeeded in using morphological markers to differentiate six species of *Urochloa* (*U. brizantha*, *U. humidicola*, *U. decumbens*, *Urochloa jubata*, *U. ruziziensis* and *Urochloa dictyoneura*) using vegetative and reproductive characteristics and pubescence. However, there is still no standard for varietal discrimination of *U. brizantha* cultivars, which can result in commercial seed lots with high varietal mix due to technical difficulties to identify the degree of contamination (Zanine et al., 2007).

For the seed production and selling of *U. brizantha*, the minimum seed purity percentage is 60% and contamination with other cultivars, must be below 0.15% (Brazil, 2008). Therefore, techniques for discrimination and for varietal purity are important for quality control in many points of the production chain, because of the easy identification for synonyms cases and homonyms between cultivars and to assist in individual selection that can be used to compose germplasm banks or in breeding plans (De Paula et al., 2012). Besides the aspect related to seed quality control, another reason for the significant increase is the interest in the cultivar characterization and identification for protection of commercial cultivars in an increasingly competitive markets.

Among the available molecular methods, those based on the polymerase chain reaction (PCR) has advantages over the other methods because they use reduced quantities of DNA and electrophoretic profiles are obtained faster (Ferreira and Grattapaglia, 1998; Ramos et al., 2006). The development of variants of this technique enabled the emergence of various types of molecular markers, including inter-simple sequence repeat (ISSR). This type of marker has been widely used in studies for varietal identification, and also in the seed technology, being a simple and efficient technique to generate high levels of polymorphism (Reddy et al., 2002). ISSR molecular markers were developed by Gupta et al. (1994) and Zietkiewicz et al. (1994). The amplified products of the PCR reaction produced in the ISSR reaction correspond to sequences of different sizes that are located between identical microsatellite repeat regions and oriented in opposite directions. In addition to presenting high levels of polymorphism, they are robust due to the fact that they have greater anchoring surface and also higher temperatures reassociation, thus having high reproducible products (Lin et al., 2010; Haq et al., 2011).

The advantages of using the ISSR technique is that it requires small DNA amounts per reaction, the speed to obtain relevant genetic information for studies of population diversity is low, and also they require little infrastructure equipment for laboratories, when compared with other markers (Satya et al., 2012) and seed can be used as a DNA source, even if the extracted amounts are low, but the quality is satisfactory. ISSR have been widely used to detect the intraspecific polymorphisms in plants (Pharmawati et al., 2004). ISSR can be used to analyze multiple loci in a single reaction (Marotti et al., 2007) and to produce fragments with high reproducibility as compared to other non-specific PCR-based markers such as random amplified polymorphic dna (RAPD) (Wolfe and Liston, 1998). Saini et al. (2004) obtained in rice, the highest percentage of polymorphic ISSR markers as compared to amplified fragments length polymorphism (AFLP) markers, but smaller than simple sequence repeats (SSR). Azevedo et al. (2011) investigated the genetic variability within genotypes of *U. ruziziensis* by ISSR. These markers are shown to be effective for such evaluation, suggesting that the populations of this *Urochloa* species retain a wide genetic variability. In the case of *U. brizantha*, despite the commercial importance of this species as a forage plant, there are still relatively few studies on the genetic diversity within existing accesses in germplasm collections in Brazil. Also, unlike *U. ruziziensis*, most commercial cultivars, *U. brizantha* have an apomictic reproduction which justifies the lowest variability among the few available accesses.

Some economically important crops have been

Table 1. List of *U. brizantha* cultivars used in this study.

Cultivar	Register Date	Maintainer	Protected for
*Basilisk	10/05/1999	MATSUDA	Public domain
Marandu	10/05/1999	EMBRAPA	Public domain
Xaraés	11/09/2001	EMBRAPA/GERMISUL/MATSUDA	Public Domain
BRS Piatã	23/06/2003	EMBRAPA	EMBRAPA
MG-4	10/05/1999	MATSUDA	Public domain
MG-5 Vitória	22/03/2000	EMBRAPA/GERMISUL/MATSUDA	Public domain

*Wrongly identified as *U. decumbens* cv. *Basilisk* (Renvoize et al., 1996).

evaluated with these markers in accesses and wild species, to obtain data for breeding assisted programs as in Silva et al. (2011) that used ISSR markers to identify inter-and intraspecific variability in accessions of *Manihot* (*Manihot esculenta*, *Manihot caerulescen*, *Manihot dichotoma* and *Manihot flabellifoli*). Also, several studies used ISSR markers as a tool for the delimitation of plant species (Dogan et al., 2007; Wood and Nakazato, 2009; Anand et al., 2010).

The DNA molecular markers have been used to discriminate cultivars and evaluate their genetic purity in seed samples. Due to the advances in molecular marker techniques, and the greater demand of the seed market, these tools have become a viable alternative for routine analysis. The ability to access the genetic variability directly from DNA has increased the process of intellectual protection of genetic materials, in association with other discriminatory analysis (Almeida et al., 2009, 2011). While in Brazil, the cultivar registration do not require molecular characterization, in other countries, with consolidated certification systems of plant material, molecular techniques have allowed developing a varietal plant molecular fingerprint, which facilitates the control of the quality at every stage in the process (Bianchi et al., 2004; Wickert et al., 2007; De Paula et al., 2012).

As exemplified above, the use of ISSR has specific characteristics to be explored in genetic analyses and varietal discrimination. Thus, the present study aimed to investigate the potential of using ISSR to discriminate the six cultivars of *U. brizantha* available and to check if this type of molecular marker could potentially determine the degree of varietal contamination among seed lots of this species.

MATERIALS AND METHODS

Genetic seeds of cultivars, *U. brizantha* Marandu, BRS Piatã, Xaraés, Basilisk, MG4, MG5 (Table 1) were provided by EMBRAPA - Campo Grande (MS) and Matsuda Seeds. MG-13 Braúna was not analysed because it was released after the start of this work.

DNA extraction and PCR

After the removal of the glumes, DNA was extracted from seeds of each cultivar as in Ambiel et al. (2008, 2010). The pellet obtained was then washed twice with 100 µl of 70% ethanol and after drying, it was resuspended in 100 μl^{-1} 1× TE buffer (2.5 mM Tris-HCl and 0.25 mM EDTA). The DNA quantification was performed in a spectrophotometer (Eppendorf BioPhotometer Plus), measuring the absorbance at a wavelength of 260 and 280 nm. The integrity of the purified DNA was assessed by electrophoresis in a 0.8% agarose gel using ethidium bromide as stain.

Initial selection was performed with 28 ISSR primers (Table 2), whereas only ten of these were useful for determining the cultivars electrophoretic pattern. PCR reactions were performed in a final volume of 25 µl containing: 25 ng of DNA, 2.5 µl (10×) *Taq* buffer (Invitrogen), 2 µl $MgCl_2$ 50 mM, 0.25 µL dNTPs (1.25 mM), 0.5 µM of each primer (Fermentas) and 2.5 U of *Taq* polymerase. The amplifications were performed in a thermocycler (PCT - 100, MJ Research) programmed for one cycle at 94°C for 1.5 min for initial denaturation, followed by 40 cycles at 94°C for 40 s, 45°C for 45 s, and 72°C for 1.5 min, the final cycle at 94°C for 45 s, 44°C for 45 s and 72°C for 5 min for final extension or elongation.

Separation of the amplified fragments was performed in a 1.2% agarose gel with 0.5 × SB buffer (10 mmol L^{-1} NaOH, adjusted to pH 8.5 with boric acid) (Brody and Kern, 2004). Gels were stained with ethidium bromide and visualized using Electrophoresis Analysis System (Biosystems). Each amplification reaction was repeated at least twice and only clearly distinct and reproducible bands were scored. The analysis of the bands was performed with the Quantum program - Capt (Vilber -Lourmat) to determine the electrophoretic pattern of the six *U. brizantha* cultivars.

Discrimination and varietal purity analysis

To test the potential of ISSR markers to discriminate the *U. brizanta* cultivars, the pattern of amplified products were tabulated as presence (1) or absence (0) for each polymorphic fragment. One genetic similarity matrix was generated using the Jaccard coefficient and a dendrogram constructed by clustering method unweighted pair-group method using arithmetic averages (UPGMA) using the software NTSYS 2.1 (Rohlf, 2004).

In order to simulate different levels of contamination varietal, a simulation of DNA contamination was performed on Marandu extracted DNA with DNA from the other cultivars in 1, 2.5 and 5% concentration. This experiment was performed in triplicate, with extraction of DNA from three samples of each studied cultivar.

After that, a PCR was performed with polymorphic primers that discriminated the Marandu cultivar from the other cultivars (P4, P7, P9, P10, P15, P23 and P24). The PCR products, of intentionally contaminated DNA samples, were separated on 1.5% agarose gel to verify the possible presence of the band belonging to the contaminant cultivar. The analyses were performed using the program Quantum - Cap, as mentioned above.

Table 2. ISSR primer nucleotide sequence used in this study.

Primer	Sequence (5 '- 3')	Primer	Sequence (5 '- 3')
P1	(CT)8 RG	P18	(GT)6 YR
P2	(AT)9 T	P19	(GT)6 AY
P3	(AG)8 G	P20	CAA(GA)4
P4	(GA)8 T	P21	(GT)6 YG
P6	(CT)8 A	P22	(GAG)4 RC
P7	(AC) 8 CT	P23	(AG)6 YC
P8	(TG)8 GG	P24	(GA)6 RG
P9	(AG)8 GYT	P25	(CA)7 YG
P10	(GA)9 YC	P26	(CTC)4 RC
P11	(GA)8 YG	P27	(GT)6 RG
P12	(CT)8 RA	P28	(GTG)4 RC
P13	(CT)8 RC	P29	(CA)7 YC
P15	(CA)6 RY	P30	(CAC)4 CCRC
P16A	(CA)6 RG	P31	GGGC(GA)6

R= (G or A); Y= (C or T).

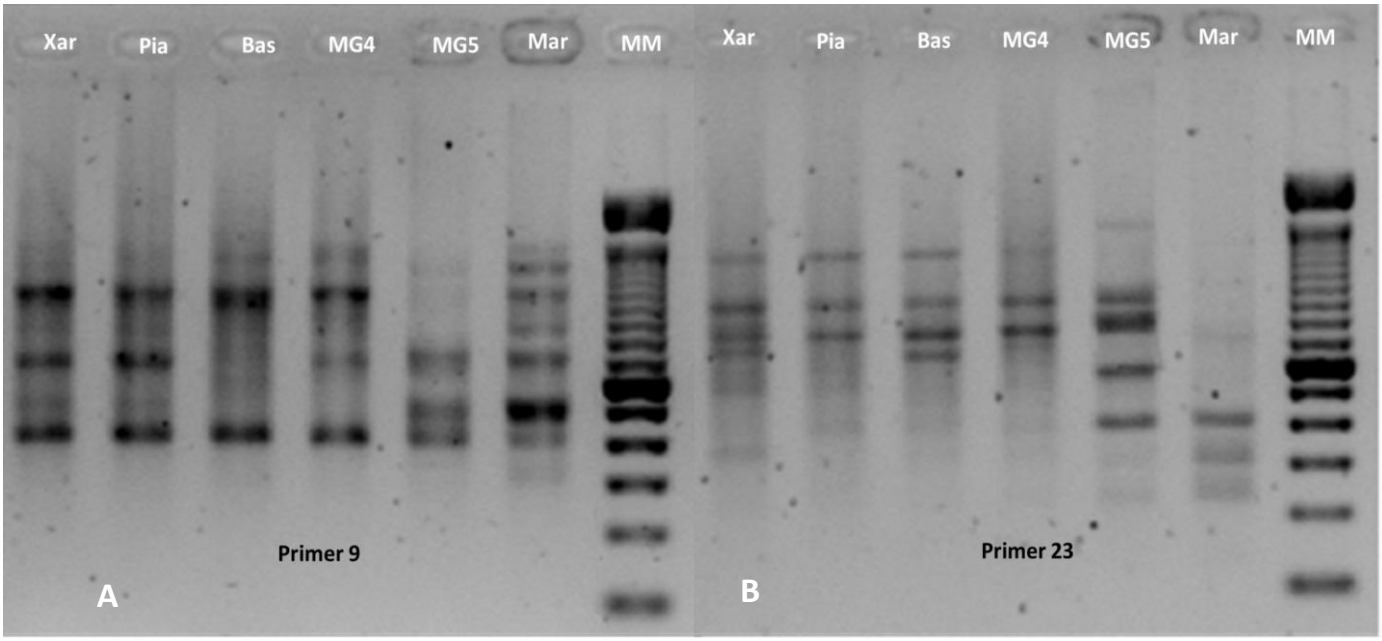

Figure 1. Example of polymorphisms of *U. brizantha* genotypes: *Xaraés* (Xar), BRS *Piatã* (Pia), *Basilisk* (Bas), MG4, MG5 and *Marandu* (Mar) and molecular markers (MM) using primers P9 (A) and P23 (B).

RESULTS AND DISCUSSION

Discrimination of cultivars

In this study, 28 ISSR primers were tested in six *U. brizantha* cultivars (Xaraés, Basilisk, Piatã, MG4, MG5 and Marandu), and 10 primers produced polymorphic bands (an example of two primers, P9 and P23 is shown in Figure 1A and B). Using these primers, a total of 65 bands were generated ranging from 120 to 2274 bp, and 10 of these were polymorphic (Table 3). From the ISSR amplified bands, a dendrogram was constructed. The grouping analysis was done using the UPGMA algorithm, by which it was possible to identify the similarity of *U. brizantha* cultivars (Figure 2). Despite the high similarity coefficient between the genotypes, some cultivars showed genetic differences. The Xaraés and MG5 cultivars have the same origin (CIAT 26110) and record

Table 3. Primer fragment length, number of bands (total and polymorphic) used in ISSR analysis of *U. brizantha* cultivars.

Primer	Fragment length (bp)	Number of bands	
		Total	Polymorphic
P4	813-1889	4	2
P7	380 -1256	7	1
P9	430 – 2274	7	1
P10	163 -1663	8	1
P15	423 – 2129	5	4
P16A	113-433	3	2
P20	140-1664	14	7
P23	120-240	4	2
P24	140-555	4	1
P26	309-1538	9	7

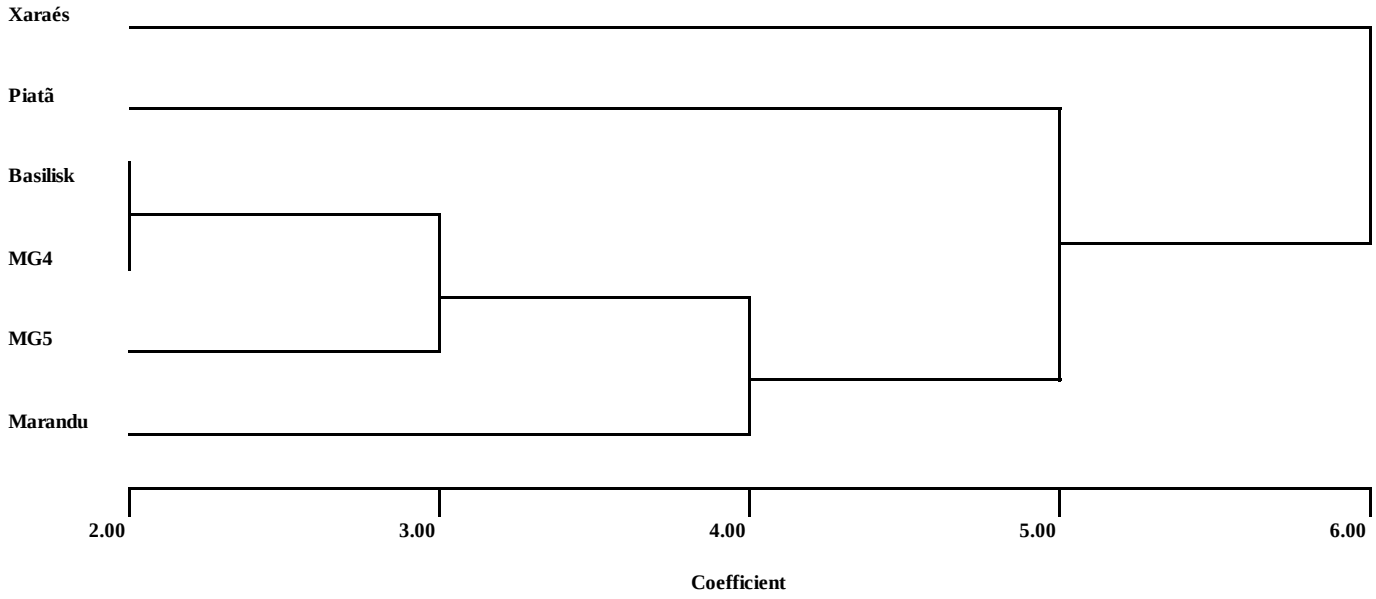

Figure 2. Grouping of six cultivars of *U. brizantha* by UPGMA method using polymorphic ISSR.

(04509) in the National Register of Cultivars (RNC)– being maintained by Embrapa and Matsuda. However, this data show that these cultivars have some genetic divergence (Figure 2), which was also verified by Ambiel et al. (2008, 2010) using the RAPD markers.

Also, it was observed that the cultivar Basilisk, erroneously classified as a *U. decumbens* genotype (Renvoize et al., 1996) was grouped with *U. brizantha* cultivars, which proved to be very similar to cultivar MG4, based on the polymorphisms obtained with the selected ISSR primers. These results confirm what was observed by others using RAPD markers (Ambiel et al., 2008; Almeida et al., 2011). Renvoize et al. (1996) suggested that the cultivar 'Basilisk', widely used and commonly identified as *U. decumbens* is actually a cultivar of *U. brizantha*. So, this error of taxonomic identification was also confirmed in this study using ISSR markers. It is important to mention that it was possible to identify and distinguish all cultivars of *Urochloa* studied in this work using the 10 selected ISSR primers.

Ten primers were used to discriminate the cultivars, but a single primer that was sufficient *per se* to identify all cultivars was not found. P4, P10 and P24 primers shown to be polymorphic among Marandu and MG4, P7 primer differentiates Marandu and Xaraés, the P9 and P23 primers discriminates Marandu from MG5 (Figure 1A and B), the P15 primer differentiated Marandu from BRS Piatã while the primer for P23 was polymorphic for Marandu and Basilisk (Figure 1B). Thus, even with the low variability among commercial cultivars *U. brizantha*, this

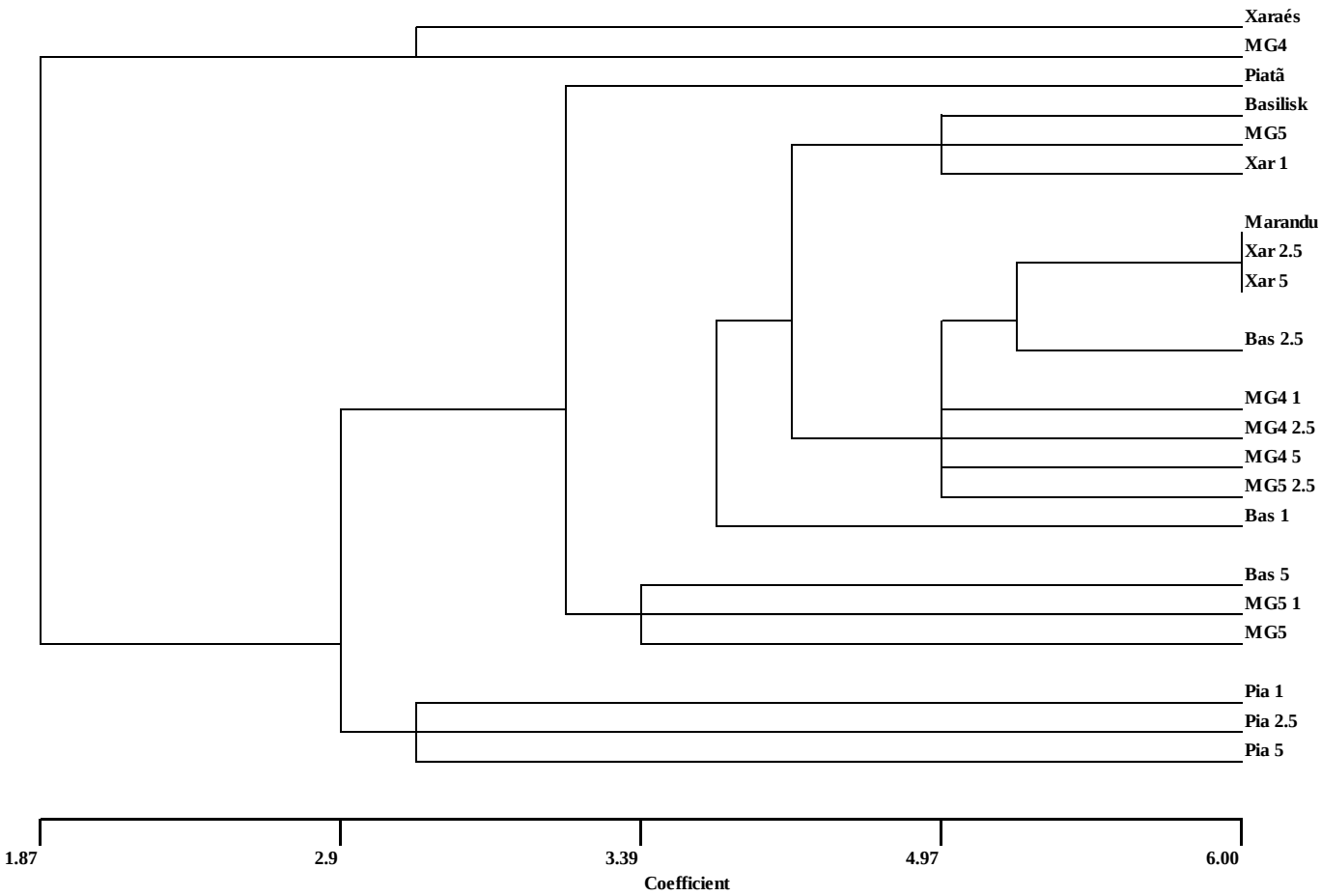

Figure 3. Grouping of *U. brizantha* genotypes: Xaraés (Xar), BRS Piatã (Pia), Basilisk (Bas), MG4 and MG5 contaminated with Marandu DNA at 1, 2 and 5%.

work showed that the use of ISSR primers allowed differentiation between these genotypes.

Silva et al. (2011) used ISSR molecular markers for the genus *Manihot,* which allowed the observation of highly reproducibility markers, revealing large intra- and interspecific divergence among accessions of *Manihot* species. Also, in studies with orchids, phylogenetic analysis showed that for genetic diversity, ISSR can respond optimally to evolutionary issues in complex species consistently and with high reliability values (Rodrigues et al., 2015).

Vigna et al. (2011) developed 15 microsatellite markers to study the genetic variability within *U. brizantha* in a germplasm collection and observed that this collection does not display a considerable variability. Thus, this work showed that the use of ISSR markers can complement the information obtained with other markers.

Genetic purity of seed lots

The second objective was to investigate the ISSR potential as markers to determine the degree of contamination of seed batches of *U. brizantha*. For this analysis simulation, a contamination of 1, 2.5 and 5% of DNA from each genotype with *U. brizantha* cultivar Marandu was made.

Several authors have studied levels of varietal purity in commercial species. Jorgensen et al. (2007) analysed the purity in batches of *Brassica napus* by ISSR, and their results indicated that there was the possibility of separating the varieties with these markers and to estimate the purity in most cases. These authors observed that in harvested seed analysed, three cultivars were contaminated with other varieties over the allowed limit (0.03% for food and <1% for feed).

The ISSR markers used in this study to explore cross-contamination with DNA, between cultivars were not efficient for determining the required levels of contamination (Figure 3) where the samples mixed with Marandu DNA did not group together; just the contaminated samples of Piatã grouped in just one cluster, but apart from the pure sample, the others are mixed with another samples or with the pure material.

According to Brasil (2008), for the purity analysis of *U. brizantha* (Hochst. Ex A. Rich) Stapf., the maximum allowed level of other cultivar at every 180 g of seeds is 30 seeds (Brasil, 2008 and 2011), for example between 0.11 and 0.13%, therefore less than 1% (considering the average number of seeds in a *U. brizantha* seed gram ranging between 123 and 145, approximately) (Brasil, 2009). In this work, even with levels higher than allowed, 5% (Figure 3), ISSR primers were able to differentiate the contamination but not identify it. Therefore, this PCR showed no efficiency in determining the contaminants from the cultivars in any tested level of contamination.

Conclusions

ISSR molecular markers proved to be useful for varietal identification of *U. brizantha* cultivars and may determine genetic differences between commercially used cultivars. The ISSR markers were not efficient to determine contamination levels at 1, 2.5 and 5% DNA between genotypes of *U. brizantha*. It was possible to reconfirm the cultivar identification of Basilisk as a cultivar of *U. brizantha.*

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Almeida CMA, Lima SN, Lima GSA, Brito JZ, Donato VMTS, Silva IMV (2009). Caracterização molecular de cultivares de cana-de-açúcar utilizando marcadores ISSR. Ciênc. Agrotec. 33:1771-1776.

Almeida MCC, Chiari L, Jank L, Valle CB (2011). Diversidade genética molecular entre cultivares e híbridos de *Brachiaria* spp. e *Panicum maximum*. Ciênc. Rural 41:1998-2003.

Ambiel AC, Machado Neto NB, Guaberto LM, Vanderlei TM (2008). Agrupamento de acessos e cultivares de três espécies de Brachiaria por RAPD. Acta Scient. Agron. 30:457-464.

Ambiel AC, Machado Neto NB, Guaberto LM, Vanderlei TM (2010). *Brachiaria* germplasm dissimilarity shown by the RAPD markers. Crop Breed. Appl. Biotechnol. 10:55-64.

Anand KK, Srivastava RK, Chaudhary LB, Singh AK (2010). Delimitation of species of the *Astragalus rhizanthus* complex (Fabaceae) using molecular markers RAPD, ISSR and DAMD. Taiwania 55:197-207.

Assis GML, Euclydes RF, Cruz CM, Valle CB (2003). Discrimination species of *Urochloa* based on different groups of morphological characters. Rev. Bras. Zootec. 32:576-584.

Azevedo ALS, Costa PP, Machado MA, De Paula CMP, Sobrinho FS (2011). High degree of genetic diversity among genotypes of the forage grass *Brachiaria ruziziensis* (*Poaceae*) detected with ISSR markers. Genet. Mol. Res. 10:3530-3538.

Bianchi VJ, Fachinello JC, Schuch MW, Sansavini S (2004). Molecular characterization of cultivars of peach and nectarine with microsatellites. Rev. Bras. Frutic. 26:490-493.

Brasil (2008). Ministério da Agricultura e Reforma Agrária. Instrução Normativa nº 30, de 21 de maio de 2008.

Brasil (2009). Ministério da Agricultura e Reforma Agrária. Regras para análise de sementes. Brasília, DF, CLAV, 2009. 365p.

Brasil (2011). Ministério da Agricultura e Reforma Agrária. Instrução Normativa nº 30, de 9 de Junho de 2011.

Brasil (2016). Ministério da Agricultura e Pecuária. Registro Nacional de Cultivares. Available at: <http://extranet.agricultura.gov.br/php/snpc/cultivarweb/cultivares_registradas.php >

Brody JR, Kern SE (2004). Sodium boric acid: a Tris-free, cooler conductive medium for DNA electrophoresis. Biotechniques 36(2):214-217.

Crusciol CAC, Soratto RP, Borghi E, Mateus GP (2009). Integração lavoura-pecuária: benefícios das gramíneas perenes nos sistemas de produção. Inf. Agron. 125:1-15.

De Paula LA, Bianchi VJ, Fachinello JC (2012). Molecular characterization and genetic variability among port peach grafts based on codominant markers. Pesq. Agropecu. Bras. 47:193-199.

Dogan B, Duran A, Hakki EE (2007). Phylogenetic analysis of *Jurinea* (Asteraceae) species from Turkey based on ISSR amplification. Ann. Bot. Fenn. 44:353-358.

Ferreira AME, Grattapaglia D (2012). Introduction to using RAPD and RFLP markers in genetic analysis. 3.ed. Brasilia: EMBRAPA-CENARGEN, Brasil. P.220

Gupta M, Chyi YS, Romero-Severson J, Owen JL (1994). Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple-sequence repeats. Theor. Appl. Genet. 89:998-1006.

Haq QMR, Singh U, Arif M, Zaidi N (2011). Genetic variability within *Fusarium solani* as revealed by PCR-fingerprinting based on ISSR markers. Indian Phytopathol. 61:305-310.

Jorgensen T, Hauser TP, Jorgensen RB (2007). Adventitious presence of other varieties in oilseed rape (*Brassica napus*) from seed banks and certified seed. Seed Sci. Res. 17:115-126.

Karia CT, Duarte JB, Araújo ACG (2006). Cultivar development of the genus *Brachiaria* (Trin.) Griseb in Brazil. Planaltina: Embrapa Cerrado. 57. (Embrapa Cerrado Documents, 163).

Keller-Grein G, Maass BL, Hanson T (1996) Natural variation in Brachiaria and existing germplasm collections. In: Miles JW, Maass BL and Valle CB (Eds.). *Brachiaria*: biology, agronomy, and improvement. 45 Cali, CIAT. pp. 16-42.

Lin XC, Lou YF, Liu J, Peng JS, Liao GL, Fang W (2010). Crossbreeding of *Phyllostachys* species (Poaceae) and identification of their hybrids Liao using ISSR markers. Genet. Mol. Res. 9:1398-1404.

Macedo MCM (2006). Respostas morfofisiológicas de *Brachiaria spp*. ao alagamento do solo e a síndrome da morte do capim-marandu. Embrapa Amazônia Oriental.

Marotti I, Bonetti A, Minelli M, Catizone P, Dinelli G (2007). Characterization of some italian common bean (*Phaseolus vulgaris* L.) landraces by RAPD, semi-random and molecular markers ISSR. Genet. Resour. Crop Evol. 54:175-188.

Matsuda (2016). Espécies forrageiras. Available at http://www.matsuda.com.br/

Pharmawati M, Yan G, Mcfarlane IJ (2004). Application of RAPD and ISSR markers to analyze molecular relationships in *Grevillea* (*Proteaceae*). Aust. Syst Bot. 17:49-61.

Ramos NP, Brunelli KR, Camargo LDA, Marcos Filho J (2006). Sensibilidade dos Microssatélites para determinar a pureza varietal em sementes de milho. Rev. Bras. Semen. 28:99-105.

Reddy MP, Sarla N, Siddiq EA (2002). Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphytica 128:9-17.

Renvoize SA, Clayton WD, Kabuye CHS (1996). Morphology, taxonomy, and natural distribution of *Brachiaria* (Trin.) Griseb. In. Miles JW et al. (Ed.). *Brachiaria*: biology, agronomy, and improvement. Cali: CIAT, 1996. pp. 1-17.

Rodrigues JF, van den Berg C, Abreu AG, Novello M, Veasey EA, Oliveira GC, Koehler S. (2015). Species delimitation of *Cattleya coccinea* and *C. mantiqueirae* (Orchidaceae): insights from phylogenetic and population genetics analyses. Plant Syst. Evol. 301(5):1345-1359.

Rohlf FJ (2004). NTSYS-Pc: numerical taxonomy and multivariate analysis system, version 2.1, user's guide New York: Exeter Software.

Saini N, Jain N, Jain S, Jain RK (2004). Assessment of genetic diversity

within and among Basmati and Basmati rice varieties non-using AFLP, ISSR and SSR markers. Euphytica 140:133-146.
Satya P, Karan M, Sarkar D, Sinha MK (2012). Genome synteny and evolution of AABB allotetraploids in *Hibiscus* section Furcaria revealed by interspecific hybridization, ISSR and SSR markers. Plant Syst. Evol. 298:1257-1270.
Silva KVP, Alves AAC, Martins MIS, Melo CAF, Carvalho R (2011). Variabilidade genética entre acessos do gênero *Manihot* por meio de marcadores moleculares ISSR. Pesq. Agropecu. Bras. 46:1082-1088.
Valle CB, Simioni C (2008). Melhoramento genético da Braquiária. In. Resende, RMS; Valle CB; Jank, L. *Melhoramento de forrageiras tropicais*. Campo Grande: Embrapa Gado de Corte.
Vigna BBZ, Jugmann L, Francisco PM, Zucchi MI, Valle CB, Souza AP (2011). Genetic diversity and population structure of the *Brachiaria brizantha* germplasm. Trop. Plant Biol. 4:157-169.
Vilela H (2005). Pastagem: seleção de plantas forrageiras, implantação e adubação. Aprender Fácil. Viçosa.
Wickert E, Lemos EDM, Pereira FM, Mayer M (2012). Marcadores fAFLP na caracterização de três genótipos de umezeiro selecionados como porta-enxertos para pessegueiro. Pesq. Agropecu. Bras. 42:1741-1746.
Wolfe AD, Liston A (1998). Contributions of PCR-based methods to plant systematics and evolutionary biology. In. Molecular systematics of plants II. Springer US. pp. 43-86.
Wood TE, Nakazato T (2009). Investigating species boundaries in the *Giliopsis* group of *Ipomopsis* (*Polemoniaceae*): Strong discordance among molecular and morphological markers. Am. J. Bot. 96:853-861.
Zanine AM, Dias PF, Souta M, Ferreira DJ, Santos EM, Pinto FB (2007). Uso de funções discriminantes para comparação de cultivares dos gêneros *Cynodon* e *Digitaria* quanto à produção de matéria seca e teores de macronutrientes. Arch. Latinoam. Prod. Anim. 15(4):159-164.
Zietkiewicz E, Rafalski A, Labuda D (1994). Genome fingerprinting by simple sequence repeat (SSR) anchored polymerase chain-reaction amplification. Genomics 20:176-183.