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Genetic diversity in cassava landraces grown on farms in Alta Floresta-MT, Brazil

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ABSTRACT. Brazil is considered one of the domestication centers of cassava (*Manihot esculenta*), containing a large part of the biological diversity and traditional knowledge of the species. The aim of the present study was to evaluate the genetic diversity of cassava landraces grown by farmers in the north of Mato Grosso State, Brazil, using inter simple sequence repeat (ISSR) molecular markers. The study was carried out in the municipality of Alta Floresta, MT, on farms located in two rural areas. Seventeen cassava landraces were selected. The DNA was extracted and polymerase chain reaction amplifications were performed

using 15 ISSR primers. Genetic similarity estimates were calculated using Jaccard's index and the generated matrix was used for clustering the genotypes by using UPGMA and Tocher's methods. The 15 ISSR primers amplified 120 fragments, revealing 61.67% polymorphism. The polymorphism information content ranged from 0.04 to 0.61, averaging 0.39. The most similar genotypes were AF5 and AF8, whereas the least similar were AF1 and AF16. The UPGMA clustering method formed five groups. Group I included twelve landraces, Group II contained two, and the other groups contained one landrace each. Tocher's method resulted in six groups: 12 landraces clustered in one group, and the other groups each contained one landrace. The ISSR markers proved efficient in revealing genetic diversity among the cassava landraces. The landraces grown by farmers in the two rural areas of Alta Floresta have a great variability and, thus, can be exploited in programs for breeding and preservation of the species.

Key words: *Manihot esculenta*; ISSR; Genetic resources; Germplasm bank

INTRODUCTION

The development of agriculture has provided information about the broad genetic diversity existing among species selected for domestication (Brush et al., 1981). Over the years, we have accumulated knowledge about landscape management, developed techniques adapted to the natural environment, and, thus, have been able to domesticate a large variety of species for food purposes (Balée, 2006; Clement et al., 2010). Similar to most cultivated species, the cassava (*Manihot esculenta* Crantz) is also the result of domestication. Domestication may be defined as "a man-driven selection process in which quantitative and/or qualitative traits of interest to the human species (productivity, uniformity, storage) are privileged over the reproductive success required in natural selection" (Valle, 2002).

It is estimated that thousands of *M. esculenta* varieties are cultivated by farmers all over the world (Elias et al., 2004). Brazil, one of the domestication centers of cassava, possesses a great part of the biological diversity and traditional knowledge associated with this species (Lebot, 2009). Many authors stress the importance of preserving the local diversity and maintaining traditional farming practices that significantly contribute to the increase and maintenance of genetic variability of cassava (Salick, 1995; Salick et al., 1997; Peroni et al., 1999). The broad genetic variability existing in cassava plantations presents favorable characteristics for *in situ* preservation and studies on genetic diversity and evolution. Cultivated plants, especially the landraces, represent a form of genetic resource that should be preserved and maintained, mainly in the transfer of qualitative traits (Faraldo et al., 2000).

Measures that aim to optimize the conservation and use of cassava germplasm are necessary to avoid losses and to guarantee the security of these genetic resources (Oliveira et al., 2014a). Landrace characterization studies of cassava grown on farms may be a strategy to facilitate management and increase the use of these genetic resources in breeding programs. To implement a breeding program, the first step is the characterization of the available germplasm. Many classify this as a pre-breeding activity. Molecular characterization, through

markers based on DNA amplification, is highly recommended because DNA is not affected by the environment in the short term, which provides greater reliability to results (Souza, 2001).

Among the molecular markers based on polymerase chain reaction (PCR), inter simple sequence repeats (ISSR) have stood out as an important instrument for the analysis of genetic diversity in the characterization of accessions and cultivars of several species (Charters and Wilkinson, 2000; Isshiki et al., 2008). ISSRs have proven to be an effective tool in the differentiation of species and varieties of the genus *Manihot* (Silva et al., 2011; Zayed et al., 2013; Vidal et al., 2015). The present study was conducted to evaluate the genetic diversity of cassava landraces grown by farmers in Alta Floresta, MT, Brazil, using ISSR molecular markers, with the aim to select material to compose a future germplasm bank of this species.

MATERIAL AND METHODS

Study area and survey of landraces

The study was conducted in the north region of Mato Grosso State, Brazil, in the municipality of Alta Floresta (09°52'32"S and 56°05'10"W), on farms in two different rural areas in the municipality. Field trips were first made to the farms to identify the cassava landraces grown by the farmers. Subsequently, all landraces identified as different by the farmer were selected. We disregarded genotypes with the same name (duplicate landraces). Seventeen cassava landraces were selected and sampled from 11 farms in the two rural areas (Table 1). Branches were collected from each genotype for later collection of leaves for molecular analysis.

Table 1. Code attributed to the 17 landraces of cassava (*Manihot esculenta*), common name given by farmers and local residents of the Rural Area of Alta Floresta-MT. RPC: root pulp color.

Code	Common name	Collection site	Origin	RPC
AF1	Cacau Roxa	Rural I Linha I	Alta Floresta	White
AF2	Cacau Arara	Rural II Linha I	Alta Floresta	White
AF3	Cenoura	Rural I Linha I	Alta Floresta	Yellow
AF4	Cacau Branca	Rural I Linha I	Alta Floresta	White
AF5	Cacau Pinheiro	Rural I Linha I	Alta Floresta	White
AF6	Mandioca Pão	Rural I Linha I	Alta Floresta	White
AF7	Vassourinha	Rural I Linha II	Alta Floresta	White
AF8	Branca Comum	Rural I Linha III	Alta Floresta	White
AF9	Mandioca de Ano	Rural I Linha II	Alta Floresta	White
AF10	Mandioca Eucalipta	Rural II Linha I	Alta Floresta	Yellow
AF11	Branca do Baiano	Rural II Linha I	Alta Floresta	White
AF12	Cacau Amarela	Rural II Linha I	Alta Floresta	Yellow
AF13	Amarela I	Rural II Linha I	Carlinda	Yellow
AF14	Amarela II	Rural II Linha I	Carlinda	Yellow
AF15	Mandioca de Fritar sem Cozinhar	Rural I Linha III	Carlinda	Yellow
AF16	Amarela III	Rural II Linha I	Paraná	Yellow
AF17	Amarela da Bahia	Rural II Linha I	Bahia	Yellow

Collection of leaf material

Leaves from the 17 cassava landraces were collected and identified in the field, packed in Ziploc® bags with silica gel, and taken to the Laboratory of Plant Genetics and Plant Molecular Biology at Universidade do Estado de Mato Grosso, Alta Floresta Campus. The leaf material was stored in a freezer at -20°C until DNA extraction.

DNA extraction

The DNA was extracted from approximately 100 mg of leaf tissue based on the cetyltrimethylammonium bromide protocol, described by Doyle and Doyle (1990). We made the modifications to the protocol: we increased the concentrations of polyvinylpyrrolidone from 1 to 2% and β-mercaptoethanol from 0.2 to 3% in the extraction buffer and reduced the incubation time at 65°C from 60 to 30 min. The DNA extracted was tested by 1% agarose gel electrophoresis, prepared in 1X Tris-Borate-EDTA (TBE) buffer and stained with ethidium bromide (0.6 ng/μL). DNA quantification was achieved by comparison with DNA-λ (100 ng/μL).

DNA amplification reactions and electrophoresis

Forty-five primers were tested for DNA amplification. Based on the amplification profile, 15 primers were selected that revealed satisfactory levels of polymorphic loci and high reproducibility of bands for the molecular characterization of the 17 landraces (Table 2).

Table 2. ISSR markers utilized for the molecular characterization of the 17 landraces.

Primer	Ta (ºC)	TNF	NPF	%P	PIC
UBC 811	52.8	12	10	83.33	0.59
UBC 815	52.8	9	7	77.78	0.61
UBC 828	51.3	7	4	57.14	0.43
UBC 888	49.0	6	1	16.67	0.04
UBC 835	51.0	8	5	62.50	0.49
UBC 834	49.2	10	6	60.00	0.42
UBC 891	47.0	8	4	50.00	0.34
UBC 868	50.0	6	3	50.00	0.25
UBC 844	48.6	10	9	90.00	0.40
UBC 808	48.8	7	3	42.86	0.35
UBC 857	52.0	5	4	80.00	0.44
UBC 856	51.0	9	6	66.67	0.45
TRI (GTG)	58.9	7	3	42.86	0.28
UBC 840	47.4	6	1	16.67	0.16
UBC 807	47.0	10	8	80.00	0.57
Total		120	74	58.43	0.39
Average/primer		8	4.93	58.43	0.39

Ta = annealing temperature; TNF = total number of amplified fragments; NPF = number of polymorphic fragments; %P = percentage of polymorphism; PIC = polymorphism information content.

The PCR amplifications were performed at a final 20 μL volume, containing: 5.8 μL H_2O, 2 μL DNA (± 20 ng), 2 μL 10X buffer (1 M KCl; 1 M Tris pH 8.3; 1 M $MgCl_2$; 10% Tween 20), 2 μL $MgCl_2$ (25 mM), 3 μL primer (0.2 mM), 4 μL dNTP (0.1 mM each dNTP), 1 μL DMSO, and 0.2 μL Taq polymerase (5 U/μL).

Reactions were performed on a thermal cycler (Biocycler), following the program described by Silva et al. (2011), with an initial denaturation phase at 94°C for 4 min, followed by 35 cycles at the following conditions: 30 s denaturation at 94°C; annealing for 35 s at 47°-58.9°C (depending on the primer used), and 2 min for the extension at 72°C. The final extension was performed at 72°C for 7 min.

Amplification products were separated by 1.5% agarose gel electrophoresis with 1X TBE buffer at 100 V constant voltage for approximately 4 h. After the electrophoresis, the gels were stained with ethidium bromide (0.6 ng/μL) for 15 min. Next, they were visualized on a UV transilluminator (LTB-21x26l) and photo-documented. The size of the amplified fragments was compared with that of a 100-bp molecular DNA marker ladder.

Analysis of amplified fragments

The amplified products were designated a single character representing presence (1) and absence (0). The ISSR markers were then converted to a binomial matrix (0/1). Because the ISSR marker is dominant, each band was assumed to represent the phenotype at a biallelic locus (Williams et al., 1990). Based on the binary matrix, the percentage of polymorphism and the polymorphism information content (PIC), proposed by Anderson et al. (1993), were calculated a function of the number of alleles detected, their distribution, and frequency in the studied population. PIC values range from 0 to 1, for monomorphic and highly polymorphic profiles, respectively.

The estimate of genetic dissimilarity between each pair of individuals was calculated by Jaccard's index, using the arithmetic complement. This coefficient consists of a comparison of the number of presences of common bands and the total number of bands involved, excluding the number of joint absences (Meyer, 2002).

Cluster analysis

Jaccard's index matrix was used for the cluster analysis of the genotypes, through unweighted pair group method with arithmetic mean (UPGMA) dendrogram, nearest neighbor (SL), and WARD hierarchical methods, using the GENES software (Cruz, 2006). With these data, the cophenetic correlation coefficient (CCC), stress, and distortion were calculated (Table 3). The UPGMA method was then selected, as it best explained the divergence of the studied material. The distance matrix generated by the arithmetic coefficient of Jaccard's index was also used for clustering the genotypes by Tocher's optimization method, employing the computational resources of the GENES software (Cruz, 2006).

Table 3. Results of cophenetic correlation coefficient (CCC), stress, and distortion, by the analysis from the WARD, UPGMA, and nearest neighbor (SL) methods.

	WARD	UPGMA	SL
CCC	0.51**	0.84**	0.82**
Stress (%)	-	17.85	32.88
Distortion (%)	-	3.18	49.32

**Significant at 1%, by the *t*-test.

RESULTS AND DISCUSSION

The 15 ISSR primers amplified 120 fragments in the 17 evaluated cassava landraces. The number of fragments per primer ranged from 5 (UBC 857) to 12 (UBC 811), with an average of 8 bands per primer. The minimum number of polymorphic bands (1) was found in primers UBC 840 and UBC 888, while primer UBC 811 revealed the maximum number of polymorphic bands (10) (Table 2). The markers revealed a total polymorphism of 61.67% (74 of 120 bands), with an average of 4.93% polymorphic fragments per primer (Table 2). This demonstrates the existence of genetic variability between the genotypes evaluated. Similar results were found by Vidal et al. (2015), who found a total polymorphism of 57.1%, when evaluating 22 *M. esculenta* accessions from the Germplasm Bank of Embrapa Cassava and Fruit Farming of Cruz das Almas, BA, Brazil. Silva et al. (2011), however, found greater polymorphism (89.7%) in their evaluation of cassava genotypes of different origins (Indonesia, Brazil, and Thailand).

The PIC for each marker ranged between 0.04 (UBC 888) to 0.61 (UBC 815), averaging 0.39. Primers UBC 815, UBC 811, and UBC 807 displayed the highest PIC values (0.61, 0.59, and 0.57, respectively; Table 2), thus these were the most informative loci. Botstein et al. (1980) considered molecular markers with PIC values below 0.25 as providing little information; those with values between 0.25 and 0.50 as providing a medium level of information; and those with values above 0.50 highly informative. In the present study, two primers presented a PIC lower than 25% (UBC 888 and UBC 840). Thus, these two marker can be considered to provide little information for the studied species. However, 80% of the analyzed loci showed a PIC greater than 25%, revealing a high discriminatory power, good multiplexing ability, and efficiency of the ISSR-PCR technique for studies aiming to quantify and organize of genetic variability of cassava. Lower PIC values (reaching only 0.21-0.27) were found by Vieira et al. (2010), in a study using RAPD markers, when characterizing the genetic viability of elite accessions of cassava for industrial purposes.

The genetic dissimilarity values ranged from 0.01 to 0.43 (Table 4). The least genetically dissimilar landraces were AF5 (Pine Cocoa) and AF8 (Common White), both with white root pulp color (Figure 1), whereas the most dissimilar landraces were AF1 (Purple Cocoa) and AF16 (Yellow Cassava III), with white and yellow root pulp color, respectively (Figure 2).

Table 4. Matrix of genetic dissimilarity between the 17 cassava landraces, calculated based on the complement of Jaccard's index, utilizing 120 ISSR fragments.

	AF1	AF2	AF3	AF4	AF5	AF6	AF7	AF8	AF9	AF10	AF11	AF12	AF13	AF14	AF15	AF16	AF17
AF1	0	0.23	0.18	0.21	0.28	0.33	0.27	0.27	0.29	0.27	0.25	0.23	0.29	0.34	0.39	0.43	0.32
AF2		0	0.22	0.29	0.23	0.19	0.12	0.22	0.25	0.25	0.18	0.25	0.24	0.26	0.33	0.38	0.27
AF3			0	0.19	0.15	0.22	0.23	0.14	0.16	0.18	0.16	0.17	0.19	0.30	0.27	0.37	0.30
AF4				0	0.23	0.29	0.29	0.22	0.25	0.21	0.22	0.21	0.31	0.35	0.32	0.39	0.28
AF5					0	0.07	0.19	0.01	0.08	0.19	0.04	0.19	0.18	0.27	0.19	0.33	0.28
AF6						0	0.13	0.08	0.14	0.23	0.05	0.23	0.14	0.24	0.24	0.33	0.32
AF7							0	0.20	0.22	0.25	0.15	0.19	0.19	0.26	0.33	0.38	0.31
AF8								0	0.06	0.18	0.04	0.18	0.18	0.27	0.18	0.34	0.27
AF9									0	0.22	0.08	0.16	0.20	0.31	0.22	0.37	0.27
AF10										0	0.17	0.20	0.24	0.29	0.27	0.23	0.30
AF11											0	0.20	0.17	0.28	0.20	0.32	0.27
AF12												0	0.17	0.19	0.27	0.39	0.25
AF13													0	0.16	0.31	0.36	0.35
AF14														0	0.35	0.40	0.31
AF15															0	0.27	0.32
AF16																0	0.37
AF17																	0

Figure 1. Color of pulp and cortex of root of the least genetically dissimilar landraces AF5 and AF8.

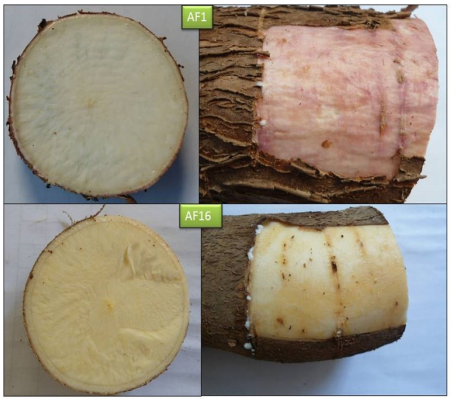

Figure 2. Color of pulp and cortex of root of the most genetically dissimilar landraces AF1 and AF16.

Of the three clustering methods tested, UPGMA was chosen for presenting the highest CCC and lowest stress and distortion values (0.84, 17.85, and 3.18, respectively) (Table 3). This method also best represented genetic diversity between the genotypes. The CCC demonstrated an association of 84% between the distances obtained in the dissimilarity matrix (complement of Jaccard's index) and the cophenetic matrix. The obtained CCC was satisfactory, given that values greater than 0.7 reflect good concordance between matrices, whereas values lower than 0.7 indicate that the clustering method is unsuitable for summarizing the information in the dataset (Rohlf, 1970). According to Cruz and Carneiro (2003), as the CCC value increases, the distortion caused by clustering individuals will also increase, which is usually obtained in the UPGMA method. Using ISSR markers in accessions of the genus *Psidium*, Oliveira et al. (2014b) obtained a CCC of 0.90 using the UPGMA method, compared to the Ward (0.80) and SL (0.85) hierarchical methods.

The results obtained by the UPGMA clustering method with the 17 cassava landraces, utilizing a cutoff point of 70%, resulted in the formation of five main groups (Figure 3). Group I was formed by 12 (AF5, AF8, AF11, AF6, AF9, AF3, AF10, AF2, AF7, AF13, AF14, and AF12) of the 17 evaluated landraces (70.59%). The two most similar landraces (AF5 and AF8) were found in this group. However, genetic variability was found within this group, since subgroups were formed (Figure 3).

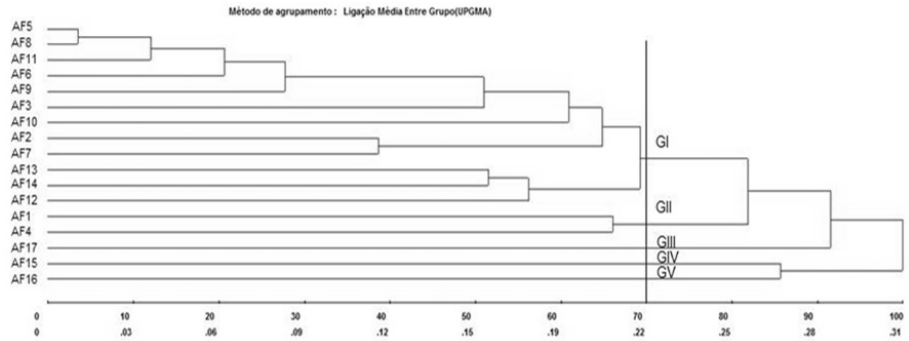

Figure 3. Dendrogram of genetic dissimilarity obtained based on ISSR markers, using the arithmetic complement of Jaccard index based on the UPGMA method for 17 cassava landraces.

Group II comprised two landraces (AF1 and AF4). It is noteworthy these two landraces, called "Cacau" by farmers from the rural areas, both show a white root pulp color. Landraces AF17 ("Amarela da Bahia"), AF15 ("Mandioca de Fritar sem Cozinhar"), and AF16 ("Amarela III") were separate from the other landraces, representing separate groups III, IV, and V, respectively (Figure 3). All three have yellow root pulp color. Ramalho et al. (2012) used ISSR markers to evaluate the genetic diversity of cassava cultivars and obtained six groups. They found that some groups were formed according to the cassava cultivars' root pulp color, which was also observed in this study, e.g., in group II.

Based on Tocher's optimization method, we identified six groups (Table 5). One group included 12 landraces, corresponding to 70.6% of the evaluated material. The other groups (A, B, C, D, E, and F) included only one landrace each (AF1, AF15, AF14, AF17, and AF16, respectively). By using Tocher's clustering method, it is common for the first group to contain the largest number of genotypes. This type of analysis is aimed at maintaining homogeneity within groups and heterogeneity between groups. Therefore, the largest number of individuals in a group indicates that they share greater genetic similarity, whereas the individuals in the last group have greater divergence in relation to those clustered in the first group (Elias et al., 2007). Based on this, landrace AF16 ("Amarela III") was the most divergent.

Table 5. Clustering by Tocher's method, based on Jaccard's dissimilarity matrix from molecular analysis by ISSR markers in 17 cassava landraces.

Groups	Cassava landraces											
I	AF5	AF8	AF11	AF6	AF9	AF3	AF13	AF7	AF12	AF10	AF2	AF4
II	AF1											
III	AF15											
IV	AF14											
V	AF17											
VI	AF16											

Landraces AF15, AF17, and AF16 ("Mandioca de Fritar sem Cozinhar", "Amarela da Bahia", and "Amarela III", respectively) were separate from other landraces by both UPGMA and Tocher's clustering methods. It is worth noting that landraces AF16 ("Amarela III") and AF17 ("Amarela da Bahia") were the only ones reported by farmers from other states (Paraná and Bahia, respectively), whereas AF15 was the only landrace reported as being "Mandioca de Fritar sem Cozinhar", which may be related to the greater divergence of these materials.

The quantification of genetic variability is one of the pillars of plant breeding. It can be used to identify genetically distant genotypes for use of distinct gene groups in crosses, to obtain superior hybrids and segregants, for evaluating the degree of genetic erosion, or to estimate the amplitude of the genetic base of cultivated forms or those under domestication and adaptation (Miranda et al., 2003; Dandolini et al., 2008; Munhoz et al., 2009). In this way, the evaluation of genetic diversity in cassava landraces is of great importance, as it is one of the pillars of the Brazilian agriculture.

We found evidence of genetic diversity among the studied cassava landraces, as several groups were formed by both clustering methods (UPGMA and Tocher's), with some material remaining isolated. The genetic diversity observed in the plantations of farmers in two rural areas, according to Oler (2012), is because of the process of exchange of materials, motivated by the desire to diversify the collection and meet possible future needs. During the visits paid to the farms, we observed the existence of an interchange of cassava landraces

among farmers from different communities, municipalities, and even states. Therefore, it is of paramount importance to know the genetic diversity of cassava kept by traditional farmers so as to preserve and maintain genetic resources.

To conclude, the cassava landraces kept and grown by producers from the rural areas of Alta Floresta, north Mato Grosso State, have a broad genetic variability that can be exploited in programs for preservation and breeding of the species. The interchange of cassava landraces grown by the farmers ensures the maintenance and divergence of the material, representing a key factor to preservation on the farms. The ethnobotanical knowledge of producers about the landraces kept on their plantations was confirmed by our molecular results, based on the corresponding divergence results.

Conflicts of interest

The authors declare no conflict of interest.

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