African Journal of Biotechnology Vol. 12(10), pp. 1040-1048, 6 March, 2013
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.2727
ISSN 1684–5315 ©2013 Academic Journals

Full Length Research Paper

Genetic diversity and population structure of sweet cassava using simple sequence repeat (SSR) molecular markers

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Accepted 1 February, 2013

The objective of this study was to evaluate the population structure and genetic diversity among 66 sweet cassava (*Manihot esculenta* Crantz) traditional accessions collected in Maringa, Parana, Brazil, using microsatellite molecular markers. Population structure was analyzed by means of genetic distances and probabilistic models; allelic frequencies were used in order to assess the genetic diversity indexes (H_t, H_o, PIC, % polymorphism and number of alleles) for each locus studied. All evaluated loci were polymorphic and the average was highly heterozygote. The number of alleles per locus was low, suggesting that restrict genetic base is a consequence of accession exchange and a reduced number of ancestors in the population. The polymorphic information content (PIC) values showed considerable genetic diversity with a mean value of 0.5076 and peak of 0.5707 for locus GA140. The microsatellites markers revealed a wide genetic variability among the traditional accessions evaluated. Moreover, the sweet cassava populations were separated in two groups using two analytical methods (probabilistic and genetic distances). The most divergent accessions were BGM 17, BGM 20, BGM 51 and BGM 95. On the other hand, the most similar accessions were BGM 25, BGM 33, BGM 37, BGM 59 and BGM 214. Hybrid combinations formed by the most divergent combinations, especially between BGM 51 × BGM 296, BGM 95 × BGM 222 and BGM 20 × BGM 12, are the most promising ones for future sweet cassava breeding programs.

Key words: Genetic diversity, *Manihot esculenta* Crantz, microsatellite markers.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) belongs to the Euphorbiaceae family and its center of origin is the central region of Brazil. The domestication center includes the regions of Tocantins, Goiás, Mato Grosso, Rondônia and Acre states (Allen, 1987; Olsen and Schaal, 1999; Olsen, 2004; Nassar et al., 2008). Cassava is an important source of food for human consumption especially in the American, African and Asian continents (Moyib et al., 2007; Raji et al., 2009).

Cassava is the third highest source of calories in the inter-tropical zone of the world (FAO, 2010) due to the high carbohydrate content in their storages roots (Ceballos et al., 2004). Also, a big portion of the

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cassava production is dedicated to the industrial sector, including biofuel processing (Kawuki et al., 2009).

Cassava has a big role in order to secure food security (Raji et al., 2009), and great part of its production in the tropical and subtropical regions is conducted by small farmers under traditional systems (Rocha et al., 2008; Zuin et al., 2009). This type of cultivation demonstrate a great advantage in the interest of preserving the genetic variability of this crop, since farmers generally select and distribute the traditional cultivars by themselves (Emperaire and Peroni, 2007; Siqueira et al., 2009; Rimoldi et al., 2010). The main characteristic of this traditional cultivar known as sweet cassava, which is common in this agriculture system, is the low cyanide content (< 100 mg HCN kg^{-1}) in the storage roots (Kizito et al., 2007).

The wide genetic variability maintained by cassava small farmers is threatened by the tendency to replace traditional crops with more attractive and better cultivars according to productivity standards, narrowing the genetic base of the crop (Vidigal Filho et al., 2000). Furthermore, the accelerate growth of cities such as Maringa (Northwestern of Paraná state, Brazil) leads to genetic erosion of traditional cassava germplasm. For this reason, the characterization of the available germplasm through population structure and genetic diversity analyses is of great interest. In past decades, molecular biology developed molecular markers techniques which are valuable tools to conduct characterization studies (Collard et al., 2005; Agarwal et al., 2008; Raji et al., 2009).

Microsatellites or simple sequence repeat (SSR*)* are currently one of the molecular markers most commonly used. These markers are highly informative due to their multi-allelic nature, co-dominant inheritance and wide genome distribution (Vignal et al., 2002; Lokko et al., 2006; Raghu et al., 2007; Raji et al., 2009). Microsatellite markers were reported to be good tools revealing polymorphism on cassava accessions (Elias et al., 2004; Lokko et al., 2006; Peroni et al., 2007; Rocha et al., 2008; Sardos et al., 2008; Siqueira et al., 2009). Therefore, the present study had the objective of assessing the population structure and genetic diversity among sweet cassava accessions collected in Maringa, Northwestern of Paraná state (Brazil) using microsatellite markers.

MATERIALS AND METHODS

Plant material and DNA extraction

A total of 66 sweet cassava accessions collected from the neighborhoods of Maringá, Paraná, Brazil, were analyzed. These accessions were provided by the Cassava Germplasm Bank of Universidade Estadual de Maringá (UEM). Accession selection was based on the previous work of Kvitschal (2008), who evaluated the genetic diversity of 75 sweet cassava accessions from Maringá using quantitative and qualitative morpho-agronomic characters. Accessions were classified as sweet cassava, since HCN content was < 100 mg.kg^{-1} in the storage roots (Kizito et al., 2007). In this case, the equivalent content of HCN, evaluated by the methodology described by Teles (1972), ranged from 40.87 to 78.94 $mgkg^{-1}$, considering the mean value equal to 61.37 mg kg^{-1} in raw pulp of storage roots. Genomic DNA extraction was performed at the Molecular Biology Laboratory from Núcleo de Pesquisa Aplicada a Agricultura - NUPAGRI, Universidade Estadual de Maringá. The extraction was conducted using a protocol described by Dellaporta et al. (1983) from young leaves (7 to 15 days old) of each accession.

Microsatellite analysis

Genomic DNA concentration was measured using FluorometerQubit® (*Qubit Fluorometer Invitrogen*) and 50 ng μL^{-1} aliquots from each sample were prepared for further amplification. Polymerase chain reaction (PCR) was performed in 25 µL total reaction mixture containing 50 ng of genomic DNA; 0.25 mM of each deoxyribonucleotide (dATP, dCTP, dGTP and dTTP); 1.5 mM of $MgCl_2$; 10 mM of Tris-HCl PCR buffer; 0.08 µM of forward and reverse primers (Invitrogen), and 0.5 unit of *Taq* polymerase enzyme (Invitrogen) and ultra-pure water (q.s.p.).

In general, the PCR amplifications were performed with a thermal cycler Techne (Model TC-512) programmed with a hot start of 95°C for 10 min followed by 30 cycles of denaturation at 95°C for 1 min. Annealing varied from 44 to 55°C for 30 s depending upon the melting temperature for the individual primer pairs (GA) and extension at 72°C for 2 min followed by final extension at 72°C for 10 min. Amplification cycles were performed according to Chavarriaga-Aguirre et al. (1998) and Mba et al. (2001) study for primers series GA and SSRY, respectively. Annealing temperature was adjusted according to the locus analyzed (Table 1).

The amplicon products were separated by electrophoresis in 10% non-denaturing polyacrylamide gels with a running time of 3 h at 150 V. A 50 bp DNA ladder (Invitrogen) was used as molecular size marker. Gels were stained with silver nitrate (Sanguinetti et al., 1994) and the DNA bands were visualized under ultraviolet light; digital images were recorded using the software Image Aide, version 3.06.04.r®.

Statistical analysis

Population structure analysis was based on Bayesian model, implemented with software Structure 2.3.3. (Pritchard et al., 2000), assuming that some fraction of each individual has been originated from the (K) populations. The most likely number of populations (K) was estimated under the admixture model and correlated allele frequencies, with no prior information on population origin (Pritchard et al., 2000; Falush et al., 2003). For this analysis, a burn-in of 100 000 Monte Carlo Markov Chain (MCMC) for each *K*, was applied, varying from *K*= 2 to *K*= 15. For each *K* value, a total of 14 clustering simulations were conducted and evaluated in relation to probabilities P(*K*) of the individuals to belong to a *K-th* group admixture model (Pritchard et al., 2000; Evanno et al., 2005; Kwak and Gepts 2009). As an aid in identifying the number of clusters of individuals (K), the results generated by Structure were subsequently analyzed with the software Structure Harvester (Earl, 2009). The threshold probability for a certain individual to belong to a *K-th* group was determined after the analysis of ΔK parameters with the software Structure Harvester (Earl, 2009).

Principal coordinate analysis (PCoA) based on a matrix of genetic distances was performed using the software GenAlEx 6.3 (Peakall and Smouse, 2006). Analysis of Molecular Variance (AMOVA) (Excoffier et al., 1992) was performed considering the *K* groups of the structure analysis. The statistical significance of the variances was tested using 10,000 random permutations at 1% probability, evaluating inter and intra groups variability. Allelic frequencies obtained with GenAlEx software confirmed the

Table 1. Microsatellite loci analyzed in the 66 sweet cassava accessions collected in Maringá, Paraná, Brazil.

Loci	LG	Motif	Primer (5´- 3´)	$MgCl_2$ (μL)	AR (bp)	AT (°C)	Ref
GA 21	nd[6]	NP[7]	F´: GGCTTCATCATGGAAAAACC R´: CAATGCTTTACGGAAGAGCC	1.25	104-126	50.0	A
GA 57	nd	NP	F´: AGCAGAGCATTTACAGCAAGG R´: TGTGGAGTTAAAGGTGTGAATG	1.25	153-183	44.0	A
GA 126	K	NP	F´: AGTGGAAATAAGCCATGTGATG R´: CCCATAATTGATGCCAGGTT	1.25	178-214	50.0	A
GA 127	K	NP	F´: CTCTAGCTATGGATTAGATCT R´: GTAGCTTCGAGTCGTGGGAGA	1.10	203-239	48.5	A
GA 134	nd	NP	F´: ACAATGTCCCAATTGGAGGA R´: ACCATGGATAGAGCTCACCG	1.10	309-337	44.0	A
GA 136	nd	NP	F´: CGTTGATAAAGTGGAAAGAGCA R´: ACTCCACTCCCGATGCTCGC	1.25	145-161	56.0	A
GA 140	nd	NP	F´: TTCAAAGGAAGCCTTCAGCTC R´: GAGCCACATCTACTGCACACC	1.25	154-164	44.0	A
SSRY 13	nd	$(CT)_{29}$	F´: GCAAGAATTCCACCAGGAAG R´: CAATGATGGTAAGATGGTGCAG	1.10	234	41.5	B
SSRY 19	V	$(CT)_{8}(CA)_{18}$	F´: TGTAAGGCATTCCAAGAATTATCA R´: TCTCCTGTGAAAAGTGCATGA	1.10	214	55.0	B
SSRY 21	B	$(GA)_{26}$	F´: CCTGCCACAATATTGAAATGG R´: CAACAATTGGACTAAGCAGCA	1.10	192	50.0	B
SSRY 45	nd	$(CT)_{27}$	F´: TGAAACTGTTTGCAAATTACGA R´: TCCAGTTCACATGTAGTTGGCT	1.25	228	55.0	B
SSRY 101	J	$(GCT)_{13}$	F´: GGAGAATACCACCGACAGGA R´: ACAGCAGCAATCACCATTTC	0.90	213	47.0	B
SSRY 135	G	$(CT)_{16}$	F´: CCAGAAACTGAAATGCATCG R´: AACATGTGCGACAGTGATTG	1.25	253	46.0	B

LG, Linkage group (Chavarriaga-Aguirre et al.,1998; Mba et al., 2001); primer: forward (F´) and reverse (R´) primers synthesized by Invitrogen; AR, amplified region (bp); AT, annealing temperature; Ref., reference: A, Chavarriaga-Aguirre et al. (1998); B, Mba et al. (2001); [6]nd, not determined; [7]NP, motif not published.

presence of low frequency alleles (f < 0.05) and rare alleles (Siqueira et al., 2009).

Genetic diversity was estimated by number, size and frequency of alleles; heterozygosity and polymorphism information content per locus (PIC), using the Power Marker software version 3.25 (Liu and Muse, 2005). The markers were classified in relation to their degree of information (PIC) according to Botstein et al. (1980) and Anderson et al. (1993). Genetic diversity among accessions was measured generating a distance matrix based on C.S. Chord's distance (Cavalli-Sforza and Edwards, 1967) with the software Power Marker 3.25 (Liu and Muse, 2005). This data was used to conduct population structure evaluation through a Neighbor-joining tree.

RESULTS AND DISCUSSION

Genetic diversity

Microsatellite analysis of the 66 sweet cassava accessions with 13 microsatellites amplified a total of 33 alleles with an average of 2.53 alleles per marker (Table 2). The presence of two alleles was observed in microsatellites GA 21, GA 57, GA 127, GA 134, GA 136 and SSRY 135, and three alleles were observed for the others primers analyzed (Table 2). The allelic frequency

Table 2. Genetic diversity indexes estimated for each microsatellite loci analyzed.

LG	Loci	Number of alleles	Allele (pb)	Frequency	PIC	H_o	Genetic diversity
			95	0.694			
Nd	GA 21	2	110	0.306	0.3347	0.6129	0.4251
			146	0.445			
Nd	GA 57	2			0.3720	0.8906	0.4940
			160	0.555			
			167	0.559			
K	GA 126	3	205	0.432	0.3833	0.6949	0.5003
			211	0.008*			
			200	0.526			
K	GA 127	2			0.3743	0.3860	0.4986
			242	0.474			
			275	0.447			
Nd	GA 134	2			0.3722	0.0526	0.4945
			280	0.553			
			57	0.518			
Nd	GA 136	2			0.3747	0.5088	0.4994
			62	0.482			
			140	0.083			
Nd	GA 140	3	145	0.492	0.4775	0.2167	0.5707
			150	0.425			
			145	0.195			
Nd	SSRY 13	3	160	0.227	0.5126	0.4688	0.5763
			201	0.578			
			140	0.070			
V	SSRY 19	3	150	0.422	0.4631	0.8906	0.5592
			160	0.508			
			65	0.008*			
B	SSRY 21	3	75	0.508	0.3860	0.8636	0.5072
			85	0.485			
			90	0.078			
Nd	SSRY 45	3	95	0.500	0.4717	0.8906	0.5659
			105	0.422			
			180	0.738			
J	SSRY 101	3	190	0.048*	0.3543	0.1111	0.4070
			195	0.214			
			125	0.500			
G	SSRY 135	2			0.3750	0.6667	0.5000
			130	0.500			
Mean		2.5385		0.5559**	0.4040	0.5580	0.5076

LG, Linkage group (Chavarriaga-Aguirre et al.,1998; Mba et al., 2001); PIC, polymorphism information content ; H_o, observed heterozygosity per locus; Nd, not determined; **mean of high allelic frequencies; *rare alleles: frequency < 0.05 according to Siqueira et al. (2009).

per microsatellite varied between 0.492 (GA 140) and 0.738, with an average of 0.556 which was considered high. The highest allelic frequency was observed for SSRY 101.

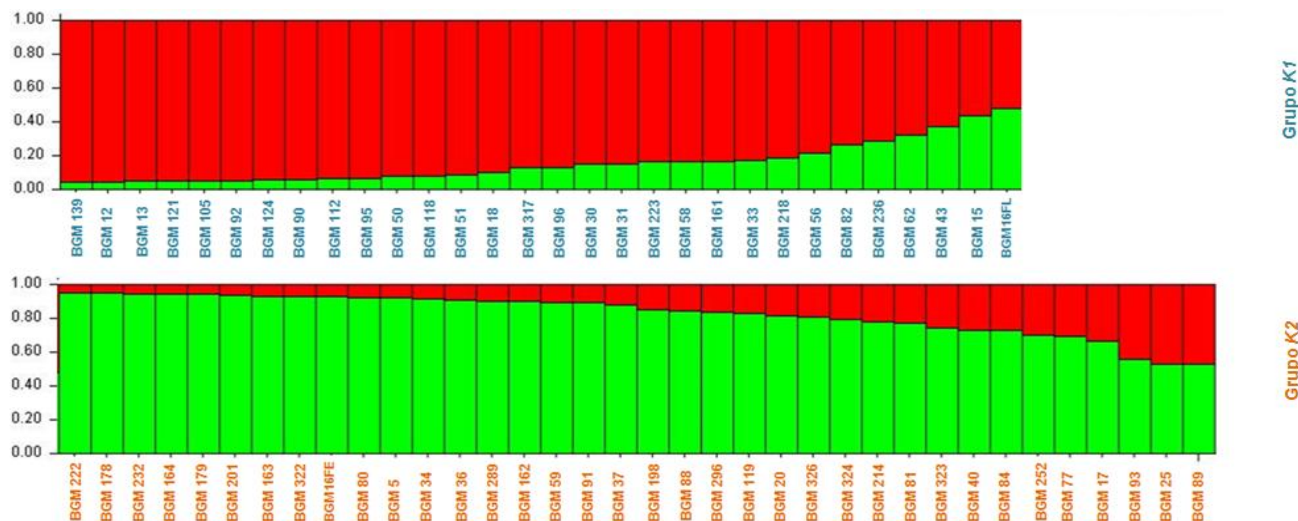

Figure 1. Population structure analysis of the 66 sweet cassava traditional accessions from Maringá, Paraná, Brazil, for $K = 2$ groups given by the software Structure 2.3.3. (Pritchard et al., 2000).

Table 2 shows the individualized allelic frequencies for each microsatellite. The presence of rare alleles ($f < 0.05$) was noted in some *loci* that have occurrence of three alleles. These rare alleles were: 211 bp in GA 126; 65 bp in SSRY 21 and 190 bp in SSRY 101. Low allelic frequencies ($0.07 < f < 0.02$) were observed for alleles 140 bp (GA 140 and SSRY 19), 145 bp (SSRY 13) and 90 bp (SSRY 45). The SSRY 101 locus showed differentiated characteristics, a rare allele, low values for heterozygosity (0.1111) and PIC (0.3543) and the highest frequency in the cluster of evaluated loci (allele 180 bp with frequency of 0.738). Therefore, these results suggest that this locus is found in a high degree of homozygosis and there is the tendency of allele fixation of 180 bp allele among the 66 sweet cassava accessions analyzed.

Another microsatellite that showed high frequency was GA 21, whose allele 95 bp was considered the second most frequent (Table 2). However, this high frequency is not reported regarding the fixation of this allele in the population, since heterozygosity of GA 21 was high (0.6129). The GA 57, SSRY 19 and SSRY 45 microsatellites were the most heterotic in the evaluated cluster with a heterozygosity value of 0.8906.

According to Table 2, the microsatellite GA 134 showed the lowest heterozygosity value (0.0526). In this case, both alleles for GA 134 tend to homozygosis without tending to fixation since the frequency of this two alleles (275 and 280 bp) are 0.447 and 0.553, respectively. Furthermore, the mean heterozygosity value observed was similar to results obtained in previous studies on cassava using microsatellite markers (Elias et al., 2004; Lokko et al., 2006; Peroni et al., 2007; Rocha et al., 2008; Sardos et al., 2008; Siqueira et al., 2009).

The genetic diversity for the accessions is shown in Table 2. The microsatellites that contributed to high rates of genetic divergence among the accessions of cassava were SSRY 13, GA 140 and SSRY 45, with values of 0.5763; 0.5707 and 0.5659, respectively (Table 2). However, the microsatellite SSRY 101 exhibited the lowest genetic diversity among the accessions with a value of 0.4070 (Table 2). As it could be noted, there are not discrepancies of the genetic diversity of loci, which means that there was a low variation in the mean genetic diversity, a fact that was also observed by Peroni et al. (2007) and Siqueira et al. (2009).

The PIC values for the microsatellites revealed a range of 0.3347 (GA 21) to 0.5126 (SSRY 13). Regarding the PIC, the most informative microsatellite marker was SSRY 13 with a PIC value of 0.5126 followed by GA 140, SSRY 45 and SSRY 19, with PIC values of 0.4775; 0.4717 and 0.4631, respectively (Table 2). Considering the series of markers used in this study, the GA 21 was the less informative with a PIC value of 0.3347. The mean value for this parameter was 0.4040 (Table 2). However, all microsatellite markers used were considered moderately informative, except for SSRY 13, which was considered highly informative according to the classification proposed by Botstein et al. (1980) and Anderson et al. (1993).

Population structure, neighbor-joining tree, and principal coordinate analysis

Population structure analysis performed using the structure software (Figure 1), further confirmed the grouping of the accessions found in neighbor-joining tree (NJ) and principal component analysis (PCoA). These analysis distributed the accessions into two distinguished

groups (K = 2), according to the evaluation of ΔK generated by the software Structure Harvester (Earl, 2009).

Only two distinct groups of cassava accessions were observed (Figure 1); the highest probability (P > 0.5) defined the group that each accession would belong to. It was confirmed that 31 accessions (47%) composed group *K1*, whereas the other 35 accessions (53%) were placed on group *K2*. Figure 1 shows the accessions allocated in group *K1* (from BGM 139 to BGM 16FL) and group *K2* (from BGM 222 to BGM 89).

The groups observed in Figure 1 showed no correlation with geographic location, since some collection sites presented accessions in both groups. Also, some accessions (BGM 15, BGM 16FL, BGM 17, BGM 25, BGM 43, BGM 77, BGM 89, BGM 93 and BGM 252) showed P(K) values between 0.5 and 0.7. The existence of a high number of shared alleles among accessions from different sites confer them a high similarity degree, as a consequence of selection and exchange of sweet cassava accessions which is a common practice by smallholders (Emperaire and Peroni, 2007; Siqueira et al., 2009; Rimoldi et al., 2010).

There are no previous reports of probabilistic methods applied on population structure analysis in cassava. The number of groups observed by population structure analysis could be used as an indicator of the real situation while other analyses, such as PCoA and NJ trees, can support these data, and make it more factual (Evanno et al., 2005; Ronfort et al., 2006; Kwak and Gepts, 2009; Efombagn et al., 2009).

A NJ tree was generated based on the genetic distance matrix of C.S. Chord (Cavalli-Sforza and Edwards, 1967) between accessions (Figure 2). Two main groups were observed with partial concordance, in terms of group members, if compared to the probabilistic method (Figure 1). According to Figure 2, it was noticed that the sweet cassava population from Maringá probably was originated from a few parental, which explains in part the high similarity among the accessions. Also, the clusters revealed a tendency to group accessions collected in close sites (Figure 2). This situation is clearly pointed out when considering accessions BGM 178, BGM 201, BGM 222 and BGM 232, all collected in the district of Iguatemi. The clustering of accessions collected in different regions, such as accessions BGM 30, BGM 105, BGM 119 and BGM 121, supports the idea of a common ancestor and the practice of material exchange among smallholders.

On the other hand, there are also divergent accessions, which were indentified according to branch length in the NJ tree in relation to the others. BGM 17, BGM 20, BGM 51, BGM 56 and BGM 296 are examples of the detected divergent accessions Furthermore, in previous studies conducted by Kvistchal (2008), BGM 17 and BGM 20 were also identified as the most divergent accessions.

Hybrid combinations formed by the most divergent combinations, especially BGM 51 × BGM 296, BGM 95 × BGM 222 and BGM 20 × BGM 12, are the most promising ones for future breeding programs. In general, these hybrid combinations are composed by individuals possessing interesting agronomic characteristics, such as starch content, which is considered high when compared to the mean of the studied population (Kvitschal, 2008) and also by the presence of flowering (facilitates the crossing in genetic breeding programs).

Moreover, in each proposed cross there is at least one individual resistant to all three diseases (root rot, bacterial blight and super elongation). Therefore, it is expected that superior segregant populations be obtained from these crosses when agronomic traits of resistance and productivity are combined.

The PCoA analysis (Figure 3) also revealed the existence of two groups, similar to the ones obtained by the probabilistic method (Figure 1). According to Figure 3, the first and the second principal coordinates were the ones that better represent the variability among the accessions.

The first two principal coordinates represent 53.25% of the total variation. The existence of similarity among some accessions can be inferred due to the proximity of accessions classified in different K groups in the thresholds zones of the groups. This fact can be verified with accessions BGM 59, BGM 121 (both belong to group *K1*), and BGM 119, BGM 162 (both belong to group *K2*), which are allocated in the center of the Figure 3. Even though the accessions were likely to belong to their respective groups due to their high probability (P(K) > 0.8), when analyzing the NJ Tree (Figure 2), the proximity among these accessions with their neighbors can also be noted.

It is important to point that the position of BGM 59, BGM 119, BGM 121 and BGM 162 in the PCoA graphic representation (Figure 3) could be explained through the origin of these cassava's traditional accessions. These samples are apparently local, with plant material being exchanged among relatives, friends and neighbors (Emperaire and Peroni, 2007; Siqueira et al., 2009; Rimoldi et al., 2010).

The AMOVA based on the molecular data showed that the groups obtained with the structure analysis (*K1* and *K2*) significantly differed from each other, with 77% of the variation remaining within groups (Table 3). These results confirm the existence of shared genotypes as a consequence of accession exchange among smallholders.

In addition, the value of minimum probabilistic of significance (MPS) reflects this high genetic variability, classifying it as very high according to Wright (1978). Therefore, it can be observed that even in an apparent unfavorable scenario, regarding the conservation of germplasm and maintenance of genetic variability, the sweet cassava population from Maringa revealed wide genetic variability among the 66 accessions evaluated.

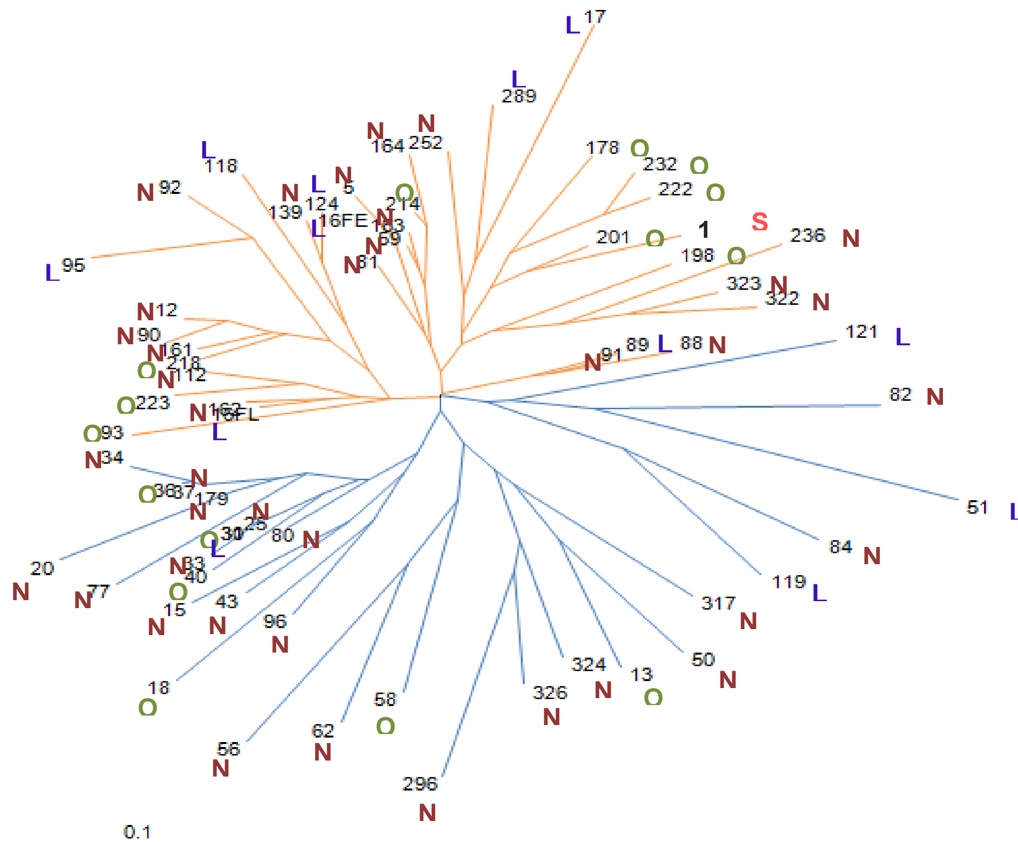

Figure 2. Neighbor-Joining tree of the 66 sweet cassava traditional accessions collected in Maringá, Paraná, according to the structure *K* groups (orange and blue). The cardinal positions were North (N); South (S); East (L); West (O).

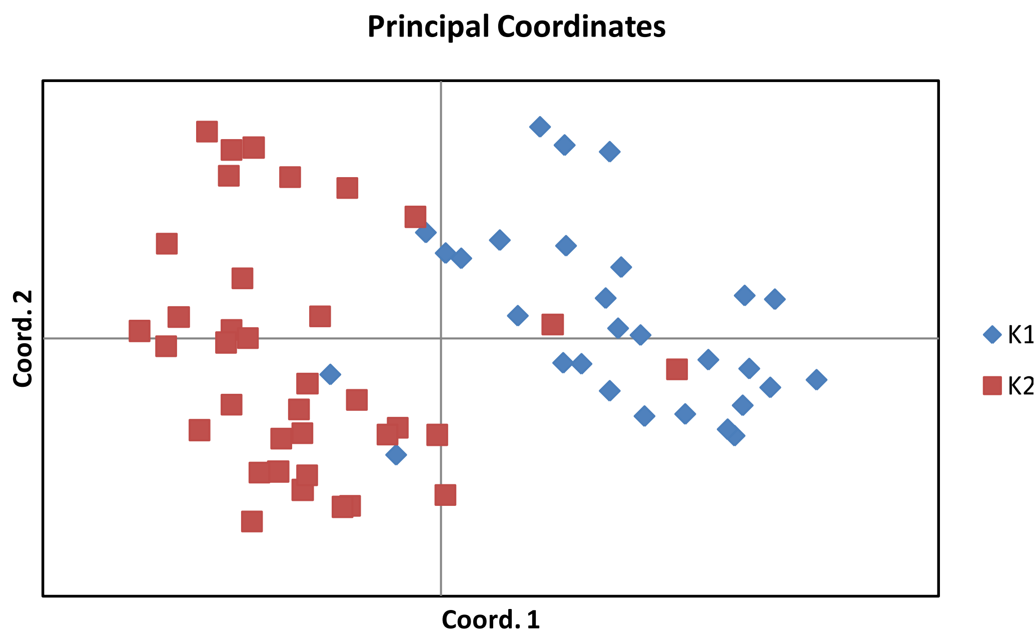

Figure 3. Comparison of population structure among 66 sweet cassava traditional accessions from Maringá, Paraná, given by the Principal Coordinates Analysis (PCoA 1 vs. PCoA 2)and by probabilistic method: *K1* and *K2* (nomenclature corresponding to structure analyses).

Table 3. Analysis of molecular variance (AMOVA) of the 66 sweet cassava accessions from Maringá, Paraná considering groups *K1* and *K2* from the structure analysis.

Variation source	DF	SS	MS	EV	%	MPS
Among K groups	1	56.121	56.121	1.554	23%**	
Within K groups	64	337.003	5.266	5.266	77%**	0.228
Total	65	393.124	61.387	6.820	100%	

DF, Degrees of freedom; SQ, square sums; MS, Mean square; EV, estimated variance; MPS, minimum probabilistic of significance; **significant at 1% of probability.

Conclusion

The results obtained in the present study reveal that the evaluated microsatellite markers were effective to characterize the genetic diversity and the population structure of 66 sweet cassava traditional accessions collected in neighborhoods of Maringá. The microsatellites markers revealed a wide genetic variability among the traditional accessions evaluated. Moreover, the sweet cassava populations were separated in two groups using two analytical methods (probabilistic and genetic distances). As a novel outcome of this study, the results of the different methods were partially in agreement in regards to accession allocation to the formed groups.

ACKNOWLEDGEMENTS

The authors would like to thank CNPq for the financial support. We would like to thank as well the Agronomy undergraduate students and Graduate students from Genetics and Breeding Program (www.pgm.uem.br) at Universidade Estadual de Maringá that were involved in the present work. M.C. Gonçalves-Vidigal and P.S. Vidigal Filho are sponsored by CNPq.

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