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Original Research Article

Molecular Genetic Diversity of Cassava (Manihot esculenta CRANTZ) in the Maritime Region of Togo using Simple Sequence Repeat (SSR) Markers

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Abstract: Cassava is a plant cultivated for its calorie-rich leaves and tuberous roots. Unfortunately, genetic improvement of cassava is limited by the lack of information on its genetic variability. The objective of the present work was to study the genetic and molecular diversity of cassava cultivars using microsatellite markers (SSR) in the Maritime region of Togo. A total of 95 cassava individuals from 7 cultivars were evaluated using 7 SSRs. The average polymorphic information content was 0.74 and the average number of alleles per locus was 7. The average observed heterozygosity was lower than the expected average heterozygosity for the 7 cultivars, showing positive fixation indices and presence of inbreeding. Molecular analysis of variance revealed greater molecular variation within cultivars (94%). The dendrogram constructed using the Neighbor Joining method resulted in 3 groups. The high genetic diversity observed could be due to the exchange of cuttings by growers. This diversity would be valuable for efficient germplasm management and for effective use of the material in breeding.

Keywords: Cassava, genetic diversity, SSR, alleles, Togo.

INTRODUCTION

Cassava (*Manihot esculenta* CRANTZ) is one of the most important food crops grown in the tropics (Phillips *et al.*, 2004). Once considered the "food of the poor", cassava has become a versatile crop that responds to the priorities of developing countries, trends in the global economy, and the challenges of climate change (FAO, 2013). The crop now plays a prominent role in the diets of more than 800 million people per day worldwide (Uarrota *et al.*, 2016). In 2021, the FAO estimated the annual global production of tuberous cassava roots at over 314.8 million tons, with Africa being the leading producer. Togo, in the same year, produced about 1.17 million tons of fresh tubers (FAOSTAT, 2021). Despite cassava's ability to adapt to different edaphoclimatic conditions due to its genetic variability (Moura *et al.*, 2013), its production is subject to numerous abiotic and biotic constraints (Akinnagbe, 2010) that result in low productivity. As a result, local cultivars with low productivity are gradually being abandoned in favour of improved varieties, leading to the erosion of local genetic resources. However, the preservation of these local cultivars is an important issue for sustainable agricultural development in Sub-Saharan Africa. In the current context, one of the strategies to combat this loss of genetic resources is to collect, analyze and organize the variability of existing clones and accessions in production areas (Djaha *et al.*, 2017).

Different methods are used in the characterization of genetic resources such as agro-morphological characterization and molecular characterization. For the last method, several genetic markers are used (RFLP, RAPD, AFLP and SSR). Among these markers, SSRs remain competitive and provide rich genetic information with good genome coverage (Kawuki *et al.*, 2013). In addition, SSRs have been widely used and have provided satisfactory results in assessing genetic diversity and population structure of cassava (Tiago *et al.*, 2017).

The general objective of this study was to provide information on the genetic and molecular diversity of cassava cultivars collected in the Maritime region of Togo using SSR markers. Specifically, the aim was to determine the genetic diversity and polymorphism of the SSR markers used, to analyze the diversity within and between cultivars and to establish the genetic structure of the individuals studied.

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1. MATERIAL AND METHODS

1.1 Sample collection

Ninety-five cassava leaf samples from seven cultivars (Table 1) were collected in the Maritime region of Togo. For this purpose, a survey was conducted in 47 cassava fields in the Maritime region. The fields were at least 10 km apart. The collected samples were placed in paper envelopes, labelled and transported from the field to the Laboratoire de Virologie et de Biotechnologies Végétales (LVBV) where molecular analyses were performed. The positions of the surveyed fields were georeferenced (Figure 1). In the laboratory, the samples were dried in an oven at 40°C.

Table 1: Distribution of leaf samples collected by cultivar

Cultivars	Number of individuals
Fetonebodji	28
Akpadjin	17
Gbaze-koute	15
Lagos	15
Yovovi he	11
Yovovi djin	5
Ganave	4
Total	95

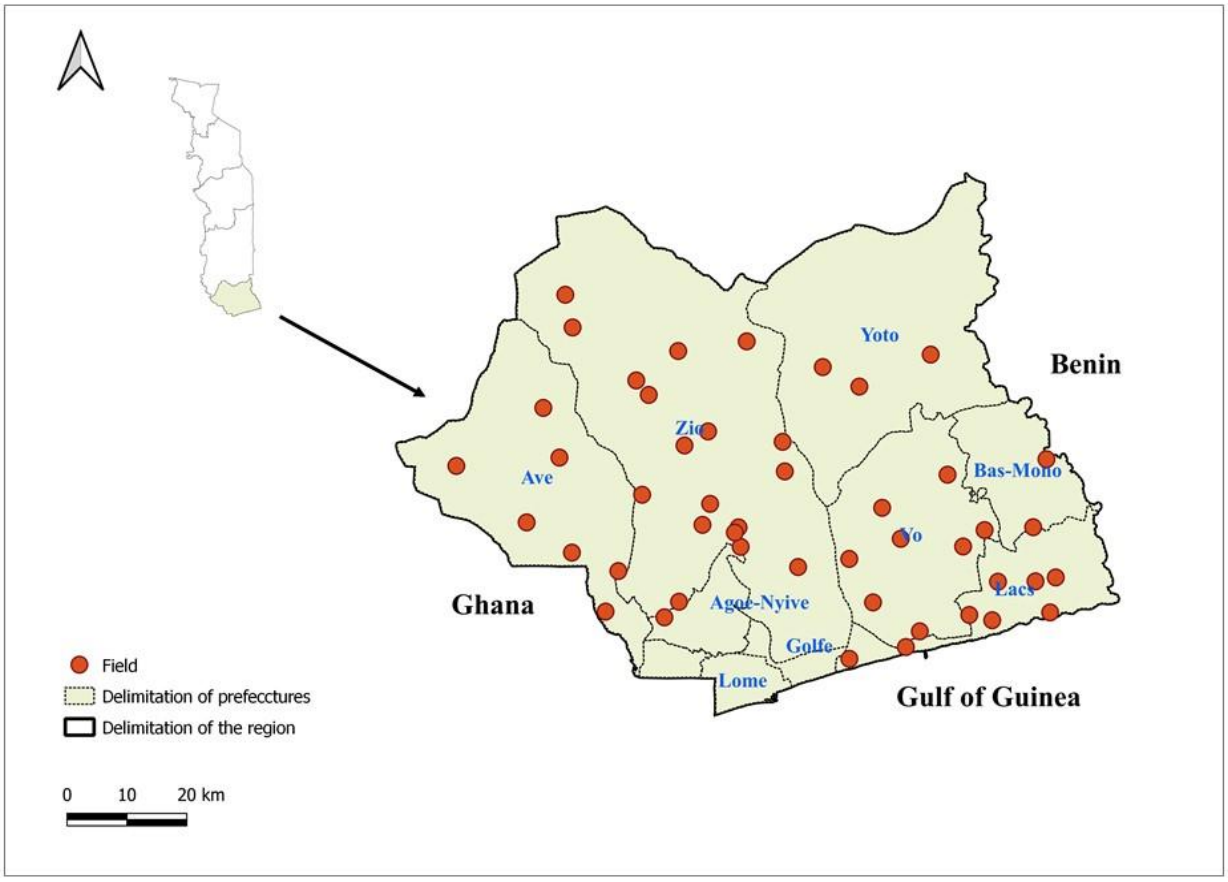

Figure 1: Geographic location of surveyed fields

1.2 Extraction of genomic DNA

DNA was extracted from 100 mg of dried and ground leaves per sample from the InnuPREP Plant DNA kit following the manufacturer's recommendations. In summary:

- 400 µl of SLS lysis solution and 20 µl of proteinase K are added to the ground leaf in a 1.5 ml tube; the whole is vortexed vigorously for 5 seconds and incubated at 65 °C for 30 min;
- The shredded material is transferred to a pre-filter and centrifuged at 11,000 rpm for 1 minute;
- 200 µl of SBS binding solution is added after centrifugation to the lysed sample and mixed and centrifuged;
- 650 µl of MS wash solution, to the spin filter and centrifuged at 11,000 rpm for 1 minute. The wash is repeated twice;

- The spin filter is placed in an elution tube to which 200 µl of elution buffer is added and incubated at room temperature for 1 minute and then centrifuged at 11,000 rpm for 1 minute.

The extracted DNA was stored at -20°C for molecular analysis.

1.3 Amplification

Seven microsatellite marker (SSR) primers, widely distributed in the cassava genome (Mba *et al*., 2001), were used (Table 2). PCR amplification was performed using the method described by Mba *et al*., (2001). Reactions were performed in a 25 µl reaction mixture consisting of the master mix (12.5 µl), sense and antisense primers (1.25 µl each), sterilized distilled water (8 µl) and DNA (2 µl). Amplification reactions were conducted in a PTC200 thermal cycler (MJ Research, Watertown, Mass).

PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide (0.5 µg/ml) in 1X TBE buffer for 90 minutes using 10 µl of PCR products. The gels were visualized and photographed in trans-UV.

Table 2: Microsatellite (SSR) primers used (Mba *et al.*, 2001)

Locus	Forward 5'……..3'	Reverse 5'……3'	Size (pb)
SSRY9	ACAATTCATCATGAGTCATCAACT	CCGTTATTGTTCCTGGTCCT	278
SSRY12	AACTGTCAAACCATTCTACTTGC	GCCAGCAAGGTTTGCTACAT	266
SSRY21	CCTGCCACAATATTGAAATGG	CAACAATTGGACTAAGCAGCA	192
SSRY69	CGATCTCAGTCGATACCCAAG	CACTCCGTTGCAGGCATTA	239
SSRY100	ATCCTTGCCTGACATTTTGC	TTCGCAGAGTCCAATTGTTG	210
SSRY161	AAGGAACACCTCTCCTAGAATCA	CCAGCTGTATGTTGAGTGAGC	220
SSRY181	GGTAGATCTGGATCGAGGAGG	CAATCGAAACCGACGATACA	199

1.4 Data analysis

The following genetic diversity parameters were estimated using Power Marker software v.3.25: average number of alleles per locus (A), average observed heterozygosity rate (Ho), average expected heterozygosity rate (He), fixation index (Fis) and polymorphic information content (PIC). These parameters were calculated for each locus and averaged over all loci (Liu & Muse, 2005). Intra- and inter-population variabilities were assessed through the calculation of population genetic differentiation (Fst) and analysis of molecular variance (AMOVA) using GenAlEx 6.5 software (Peakall & Smouse, 2006). Genetic structure was established by the dendrogram constructed by the 1000 bootstrap Neighbour Joining method using DARWIN 6.0.11 software.

2. RESULTS AND DISCUSSION

2.1 Genetic diversity and polymorphism of SSR markers used

A total of 88 of the 95 leaf samples used was generated at least one allele. The remaining samples did not generate any alleles and were therefore not included in the analyses.

All 7 primers used generated polymorphic profiles. A total of 49 alleles were detected, with an average of 7 alleles per locus, ranging from 5 (SSRY9 and SSRY181) to 9 (SSRY21 and SSRY69) (Table 3). The allelic frequency of all primers was generally less than 0.95 indicating that the primers had a polymorphic character.

The average number of alleles per locus is identical to that obtained in a study in Brazil in the state of Mato Grosso (north) where 14 microsatellites markers were used to describe the genetic diversity of 120 cassava individuals (de Pedri *et al*., 2019). On the other hand, the average number of alleles obtained is higher than that obtained in Vanuati where 11 SSR primers on 104 cassava genotypes detected an average of 5.36 alleles per locus (Sardos *et al*., 2008). These results show that the number of alleles per locus varies with the number and type of microsatellites markers used and the number of individuals genotyped.

The average expected heterozygosity (He) at all loci ranged from 0.58 at the SSRY9 locus to 0.84 at the SSRY21 locus with a means of 0.77, whereas the observed heterozygosity (Ho) ranged from 0 (SSRY9) to 0.32 (SSRY100) with a means of 0.15 (Table 3). The observed heterozygosity on all seven markers was lower than the expected mean heterozygosity, indicating the predominance of homozygotes and thus positive values for the fixation index.

In this study, high genetic diversity was observed among the 88 genotyped individuals. The He was the same (high) in the study conducted by Sousa and colleagues in 2017 on cassava where the He was 0.68 (Sousa *et al*., 2017). These results could be explained by the exchange of cassava cuttings among neighbors, relatives and friends; which is very usual in traditional farming systems (da Costa *et al*., 2013).

The polymorphic information content (PIC) values calculated for the 7 loci are quite high with an average of 0.7. The highest value in this study was obtained for the SSRY21 locus (0.82) and the lowest value was recorded for the SSRY9 locus (0.56) (Table 3).

Table 3: Estimated genetic diversity by locus

Locus	Allelic frequency	Number of individuals	N_a	H_e	H_o	Fis	Fst	PIC
SSRY9	0.58	59	5	0.60	0	1	0.126	0.56
SSRY12	0.35	71	6	0.78	0.10	0.87	0.040	0.75
SSRY21	0.25	53	9	0.85	0.09	0.89	0.032	0.83
SSRY69	0.34	56	9	0.82	0.30	0.63	0.044	0.80
SSRY100	0.24	33	7	0.82	0.33	0.60	0.019	0.80
SSRY161	0.30	67	8	0.78	0.15	0.81	0.075	0.74
SSRY181	0.28	29	5	0.78	0.03	0.96	0.071	0.74
Multilocus	0.33	88	7	0.77	0.14	0.81	0.058	0.75

Na = number of alleles, He = expected heterozygosity, Ho = observed heterozygosity, Fis = fixation indices, Fst = genetic differentiation, PIC = polymorphic information content.

According to Botstein *et al*., (1980), the information content of the polymorphic information content (PIC) is used to estimate the quality of molecular marker detection of polymorphism in individuals, and it can be classified as satisfactory (PIC > 0.5), medium ($0.25 \leq P \leq 0.5$), and low (PIC < 0.25). The PIC values obtained are a testimony to the effectiveness of the markers used and are classified as satisfactory. This is consistent with other studies carried out on cassava, where the majority SSR tested have been highly polymorphic with PIC values ranging from 0.293 to 0.821 (Turyagyenda *et al*., 2012; de Pedri *et al*., 2019).

2.2 Analysis of Diversity Within and Between Cultivars

The genetic diversity within the studied cultivars showed that the number of alleles varied from 6 (Ganave) to 10 (Fétonégbodji), with an average of 7.85 per cultivar. The expected heterozygosity (He) by cultivars was on average 0.55, with the lowest value 0.33 in Ganave and the highest value 0.7 in Lagos. The observed heterozygosity (Ho) was on average 0.18 per cultivar and varied from 0.08 in Yovovi djin to 0.18 in Gbazé-kouté (Table 4).

In this study, for each cultivar, He was widely above Ho, thus generating positive values for Fis, a sign of an excess of homozygotes. These results are contrary to the results from the study in Brazil where Ho was higher than He (de Pedri *et al*., 2019). On the other hand, the results obtained corroborate the results of Adjebeng-Danquah *et al*., (2020) in Ghana, where the fixation indices were positive.

Table 4: Estimated genetic diversity of cassava cultivars

Cultivars	Na	H_e	H_o	Fis	PIC
Akpadjin	8	0.62	0.15	0.75	0.56
Fetonegbodji	10	0.62	0.09	0.86	0.59
Ganave	6	0.33	0.07	0.79	0.27
Gbaze-koute	8	0.63	0.18	0.72	0.57
Lagos	8	0.7	0.16	0.77	0.65
Yovovi djin	7	0.39	0.08	0.80	0.32
Yovovi he	8	0.59	0.16	0.72	0.53
Mean	7.85	0.55	0.13	0.77	0.5

Na = number of alleles, He = expected heterozygosity Ho = observed heterozygosity, Fis = fixation indices, Fst = genetic differentiation, PIC = polymorphic information content.
He and He for Lagos were calculated on six loci, because SSRY100 did not detect any alleles for this cultivar

In accordance with Wright's F-statistics results, the highest genetic differentiation coefficient Fst value was recorded by the SSRY9 locus (0.126), while the lowest was obtained by the SSRY100 locus (0.019) (Table 3). The average differentiation (Fst = 0.058) reveals that 5.8% of the total variability is explained by variation between cultivars, while 94.2% of this variability is attributed to variation within cultivars. These F-statistics results corroborate the results of the molecular variance analysis (Table 5) and show that the majority of the diversity (94%) is located within cultivars, which implies the low differentiation (6%) of genotypes between cultivars. These results would be due to gene flow and genotype exchange between regions (Kombo *et al*., 2012). Other authors have also found a higher variation within than between cassava populations using molecular markers (Lokko *et al*., 2006; Siqueira *et al*., 2009).

Table 5: Molecular analysis of variance (AMOVA) of 7 cassava cultivars with 7 SSRs markers

Source of variation	df	SS	MS	Var. Est.	%
Between cultivars	6	35.79	5.964	0.149	6
Within cultivars	169	407.45	2.411	2.411	94
Total	175	443.24		2.560	100

df = degree of freedom; SS = sum of squares. MS = means of the sums of squares; Var.Est = estimated variance; % = percentage of variation.

The results also reveal that the studied manioc cultivars exhibit moderate differentiation according to the Wright scale (1965). This differentiation is higher than the genetic differentiation between elite accessions and local varieties in Uganda, which was 0.025 (Turyagyenda *et al*., 2012).

2.3 Genetic Structure

An evaluation of the genetic relationships between cultivars was conducted by estimating Nei and Li genetic distances (1973). The dendrogram constructed from these genetic distances using the Neighbor Joining (NJ) method separated the 88 accessions into 3 main groups (Figure 2). Group 1 (G1) is composed of 37 individuals, Group 2 (G2) of 20, and Group 3 (G3) of 31 individuals. The genetic groups thus formed did not group most individuals.

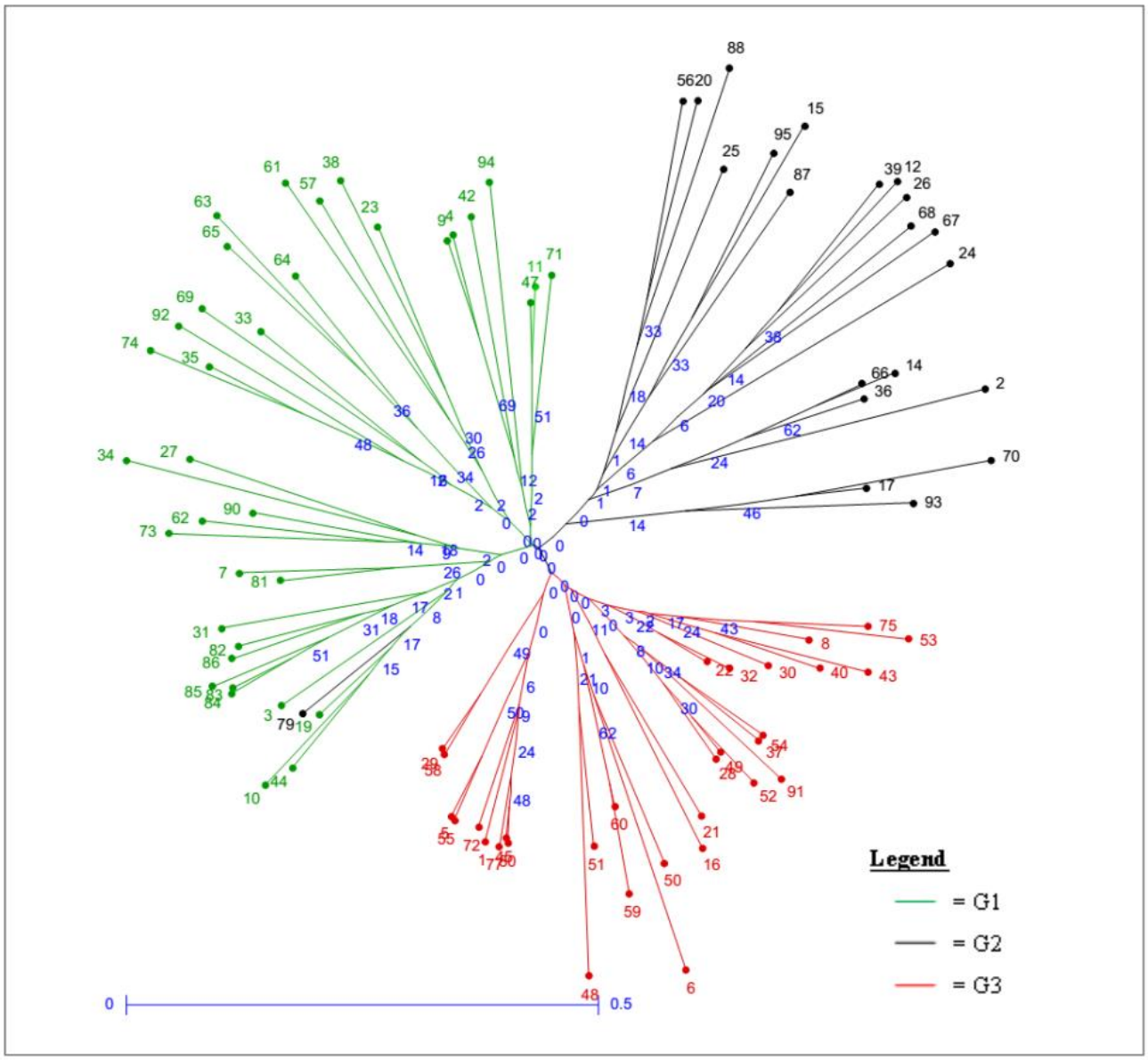

Figure 2: Genetic structuring of 88 cassava samples grown in the maritime region of Togo

The proportion of individuals distribution for each cultivar was not uniform within the genetic groups. The created genetic groups once again demonstrate the variability that exists among the studied cultivars. All individuals from the Ganave cultivar were found in Group G2 (Figure 3).

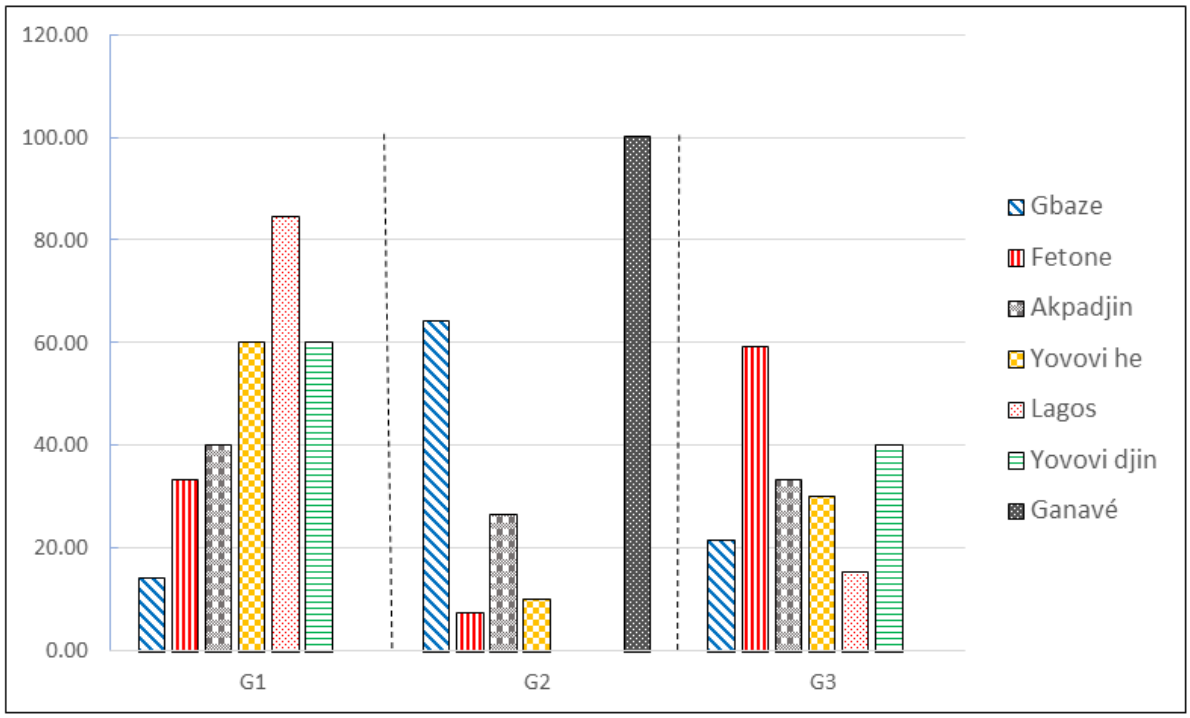

Figure 3: Distribution of cultivars within the genetic groups created

More than 80% of the individuals of the Lagos cultivar were found in Group G1, as well as 60% of the individuals of the Yovovi he and Yovovi djin cultivars. Approximately 60% of the individuals of Gbaze-koute and Fetonegbodji were found in groups G2 and G3, respectively. Individuals from the Gbaze-koute, Fetonegbodji, Akpadjin, and Yovovi he cultivars were present in all three genetic groups.

The created genetic groups once again demonstrate the variability that exists among the studied cultivars, which may be attributed to the fact that the studied populations were composed on the basis of names given by farmers. Generally, in traditional agriculture, cultivars are named differently according to locations or people when they are passed on from one person to another (Mekbib, 2007) and also based on agro-morphological traits. As a result, different cultivars may have the same name or the same cultivar may have multiple names (Elias *et al*., 2001). Therefore, names themselves are not a reliable indicator of diversity (Sardos *et al*., 2008).

3. CONCLUSION

The study of the genetic diversity of 95 cassava leaf samples using 7 SSRs markers is only a first approach in understanding the genetic diversity of cassava grown in the Maritime region of Togo. Indeed, a significant amount of polymorphic information (PIC > 0.5) was revealed for the SSRs with an average number of alleles of 7 per locus. The average observed heterozygosity per locus was low (0.14) while the average expected heterozygosity per locus was high (0.77) resulting in positive Fis values. Intra-population variability (94%) was significantly compared to inter-population variability (6%). The dendrogram constructed based on genetic distances grouped genotypes into 3 groups. The high genetic diversity observed within the studied cassava cultivars could be due to the exchange of genetic material (local varieties) carried out by farmers, who act as maintainers of local diversity.

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