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Genetic Characterization of Cashew (*Anacardium occidentale* L) Cultivars from Benin

Sika KC[1,2,3], **Adoukonou-Sagbadja H**[4], **Ahoton L**[5], **Saidou A**[5], **Ahanchede A**[5], **Kefela T**[1], **Gachomo EW**[1,2], **Baba-Moussa L**[3] **and Kotchoni SO**[1,2,*]

[1]*Department of Biology, Rutgers University, 315 Penn St., Camden, NJ, USA*

[2]*Center for Computational and Integrative Biology, Rutgers University, 315 Penn St., Camden, NJ, USA*

[3]*Department of Biochemistry and Cell Biology, Faculty of Science and Technology, University of Abomey, Benin*

[4]*Department of Genetics and Biotechnology, University of Abomey, Benin*

[5]*Department of Plant Production, Faculty of Agricultural Sciences, University of Abomey, Benin*

[*]**Corresponding author:** Kotchoni SO, Center for Computational and Integrative Biology, Rutgers University, 315 Penn St., Camden, NJ, USA, Tel: (848) 445-2075; Fax: (732) 445-5870; E-mail: simeon.kotchoni@rutgers.edu

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Abstract

Cashew (*Anacardium Occidentale L.*) is a fruit plant introduced in Benin in 1950 and is widely cultivated in private plantations in three regions (Central, North East and North West) of the country. These plantations contain different varieties of cashew with reduced productivity and quality of nuts. In this study we characterized the genetic diversity of Benin's cashew accessions by SSR (simple sequence repeat) markers. Sixty cashew morphotypes from three regions of Benin, West Africa, were analyzed using eight SSR markers. A total of 146 polymorphic bands were produced. The polymorphic bands showed low genetic diversity (Shannon index = 0.04) that are relatively important for an imported species. Low diversity was found in North western regions compared to the other two regions (Center and North East). Genetic distance-based UPGMA dendrogram and Principal Coordinate Analysis (PCoA) showed a genetic differentiation between morphotypes. This genetic differentiation allowed us to cluster the samples into three clusters based on their genetic variations. This work further provides genetic information for the improvement of cashew production, conservation and better management of *A. occidentale* genetic resources in Benin.

Keywords: SSR; PCR; *Anacardium occidentale L.*; Benin; Genetic diversity; Plant diversity; Crop production; Genetics

Introduction

Cashew (*Anacardium occidentale L*) is tree nut native to tropical South American countries [1,2]. *A. occidentale* was introduced into Africa and India in the 16th century [3], and has spread so widely that these new areas of cultivation have become the center of diversity of cashew today. Cashew is cultivated in agricultural areas degraded by extensive cultivation in sub-Sahara Africa. Therefore cashew trees are not only important in reclaiming farming land but have become one of economically important crops in countries such as Tanzania, Mozambique, Nigeria, Guinea Bissau, Côte d'Ivoire and Benin [4].

Cashew production in Benin and other countries is constrained by low and variable nut yields, nut quality and susceptibility to pests and diseases [5]. Several studies have shown that most the cashew trees growing in Africa are still in the wild state and these trees produce nuts low quality [6,7]. This is mainly due to large area planted with planting material of non-descript origin. Meanwhile, an important way to increase the productivity of the crop and improve on the quality of nuts is to select desirable genotypes from the existing varieties and use identified superior materials the breeding programs [5]. To initiate a good cashew breeding program in Benin, it is important to have knowledge on the species genetic diversity with the aim to select elite trees that can be used in breeding schemes. However, breeding of cashew is mostly based on traditional methods of selection of useful phenotypic and agronomic traits such as nut size, nut weight, color of apple sex ratio, size of the fruits, tree canopy, length of panicle and yield performance [4,8]. The cashew identification is mainly based on morphological traits. Cashew descriptor developed by International Board for Plant Genetic Resources (IBPGR) for the evaluation of cashew has been used for the morphological characterization [9-11]. Although classical phenotype features are still extremely useful, the efficiency of selection may be reduced by environmental effects on evaluated traits [5]. Dhanaraj et al. [12] reported that data obtained from such morphological evaluations are not always based on the assessment at genetic level. In such circumstance, molecular markers are useful as they are stable and unaffected by the environment to study the genetic relationships, and helps in identification of varieties.

Molecular markers are very useful for efficient management and utilization of genetic resources [13,14]. Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Simple Sequence Repeat (SSR) were used to study the diversity of cashew in Tanzania, Nigeria, India and Brazilia [2,10,15,16].

The aim of this study was to evaluate the genetic diversity of cashew cultivars grown in Benin by the molecular microsatellite markers (SSR). Their distribution in the whole genome, high polymorphism, loci specificity, co-dominance and ease of use make the molecular microsatellites excellent genetic markers [17]. In this work, well characterized Anacardium occidentale SSR markers [10,18] were used for the first time to study the genetic diversity of A. occidentale cultivars in Benin.

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Materials and Methods

Plant material

Sixty cultivars of cashew (*Anacardium occidentale L.*) used in this study were selected from different geographical locations in Benin (Table 1).

N°	ID	Site of Collection	Geographical Area	Age (years)	N°	ID	Site of Collection	Geographical Area	Age (years)
1	C1-1	Kilibo	Central	15	31	O14-3	Djougou	North West	15
2	C3-2	Kilibo	Central	15	32	O16-4	Djougou	North West	9
3	C4-1	Kilibo	Central	15	33	O17-1	Djougou	North West	9
4	C5-1	Kilibo	Central	12	34	O19-1	Djougou	North West	15
5	C6-1	Kilibo	Central	12	35	O20-1	Djougou	North West	12
6	C8-4	Kilibo	Central	9	36	O21-2	Djougou	North West	9
7	C9-3	Kilibo	Central	9	37	O24-4	Djougou	North West	12
8	C10-1	Kilibo	Central	12	38	O27-1	Bassila	North West	15
9	C11-2	Kilibo	Central	9	39	O29-1	Bassila	North West	9
10	C12-4	Kilibo	Central	12	40	O32-1	Bassila	North West	12
11	C14-2	Glazoué	Central	12	41	E2-4	Parakou	North East	15
12	C16-1	Glazoué	Central	12	42	E5-1	Parakou	North East	15
13	C17-4	Glazoué	Central	12	43	E6-1	Parakou	North East	9
14	C18-1	Glazoué	Central	9	44	E7-2	Parakou	North East	9
15	C20-1	Glazoué	Central	12	45	E9-1	Parakou	North East	12
16	C21-2	Glazoué	Central	12	46	E11-4	Bembèrèkè	North East	12
17	C22-2	Glazoué	Central	12	47	E13-4	Bembèrèkè	North East	12
18	C23-2	Glazoué	Central	15	48	E15-1	Bembèrèkè	North East	9
19	C24-1	Glazoué	Central	9	49	E18-4	Bembèrèkè	North East	9
20	C25-1	Glazoué	Central	9	50	E19-4	Bembèrèkè	North East	9
21	C26-2	Glazoué	Central	9	51	E21-2	Bembèrèkè	North East	15
22	C28-2	Glazoué	Central	15	52	E25-2	Serekale	North East	12
23	C29-1	Glazoué	Central	15	53	E26-1	Serekale	North East	12
24	C31-4	Bantè	Central	12	54	E28-4	Serekale	North East	12
25	C33-3	Bantè	Central	12	55	E31-2	Serekale	North East	9
26	C39-2	Bantè	Central	9	56	E32-2	Serekale	North East	9
27	C40-1	Bantè	Central	15	57	E34-2	Serekale	North East	9
28	C43-3	Bantè	Central	9	58	E35-2	Serekale	North East	15
29	O11-1	Djougou	North West	15	59	E37-1	Serekale	North East	15

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30	O12-4	Djougou	North West	15	60	E39-4	Serekale	North East	15

Table 1: Identification numbers, Site of collection and age of the cashew (*Anacadium occidentale* L.) genotypes used in the study.

These cultivars were phenotypically characterized using the International Board for Plant Genetic Resources (IPBGR) descriptor as previously described [11] These morphotypes were collected from three cashew-growing regions of Benin: (28 cultivars from central region; 12 cultivars from North West and 20 cultivars from North East region of the country (Figure 1).

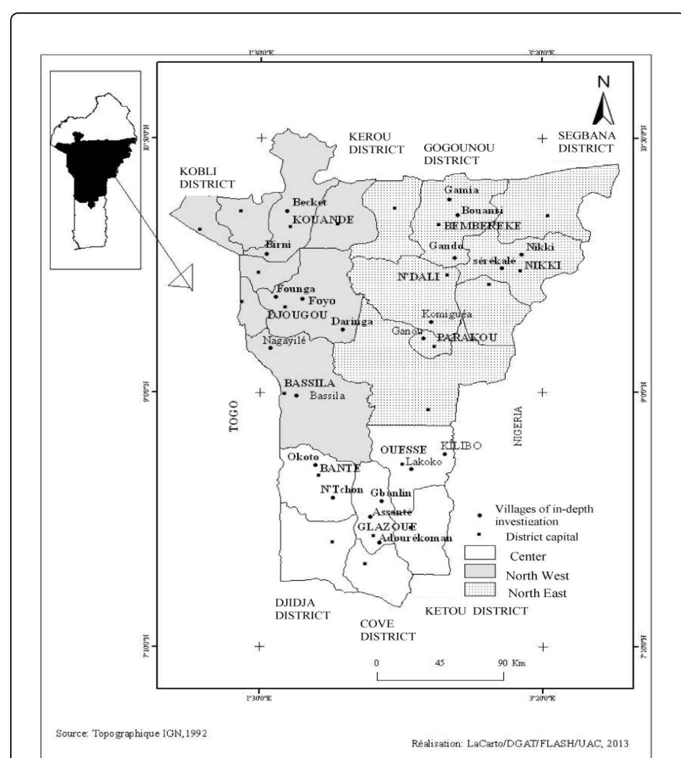

Figure 1: Locations of regions from which cashew (*Anacadium occidentale L.*) tree were collected for the genetic analysis. The genotype included in this study belonged to three geographical regions in Benin where have produced the cashew: Center (28 morphotypes); North West (13 morphotypes) and North East (19 morphotypes).

DNA extraction

Young and healthy leaves from single trees of each morphotype were used for DNA extraction according to Kotchoni and Gachomo [19].

SSR method

Eight well characterized SSR primers [18] were used for the genetic characterization of the cashew cultivars. These markers were selected in this study because they revealed great polymorphism in Nigerian cashew genetic diversity study [10]. The SSR primer pair characteristics are depicted in Table 2.

Locus	Primer sequence (5'-3')	Repeat motif	Ta (°C)	Allelic size range (bp)
mAoR3	F: CAGAACCGTCACTCCACTCC R: ATCCAGACGAAGAAGCGATG	(AC)12(AAAAT)2	60.3	241-247
mAoR6	F: CAAAACTAGCCGGAATCTAGC R: CCCCATCAAACCCTTATGAC	(AT)5(GT)12	58.2	143-157
mAoR7	F: AACCTTCACTCCTCTGAAGC R: GTGAATCCAAAGCGTGTG	(AT)2(GT)5AT(GT)5	58.2	178-181
mAoR11	F: ATCCAACAGCCACAATCCTC R: CTTACAGCCCCAAACTCTCG	(AT)3(AC)16	60.3	234-236
mAoR17	F: GCAATGTGCAGACATGGTTC R: GGTTTCGCATGGAAGAAGAG	(GA)24	56.1	124-159
mAoR42	F: ACTGTCACGTCAATGGCATC R: GCGAAGGTCAAAGAGCAGTC	(CAT)9TAT(CTT)7	60.3	197-206
mAoR48	F: CAGCGAGTGGCTTACGAAAT R: GACCATGGGCTTGATACGTC	(GAA)6(GA)3	58.2	172-178
mAoR52	F: GCTATGACCCTTGGGAACTC	(GT)16(TA)2	58.2	191-203

	R: GTGACACAACCAAAACCAC A			

Table 2: Characteristics of SSR microsatellite markers used in this study.

The DNA fragments were PCR-amplified according to the Quanta Biosciences kit recommendation. The total volume of PCR mixture was 20 µl and comprised 10 µl of master mix [AccuStart II PCR ToughMix (2x)], 4 µl template DNA, 1 µl of each primer (Forward and reverse) and 4 µl water. The PCR reaction was performed in a thermal cycler (BIO-RAD; T100TM) using an initial 94°C denaturing step for 3 min followed by 34 cycles at 94°C for 30s, annealing for 30s at the primer's annealing temperature, extension at 72°C for 1 min 20s and a final extension at 72°C for 5 min.

Data analyses

The presence or absence of a PCR amplified SSR marker band was coded 1 or 0 respectively. The data was entered in an Excel spreadsheet in order to generate the analysis matrix. Genetic diversity parameters such as Polymorphism Information Content (PIC) et al. [20], rate of polymorphism (P), number of alleles (N), expected heterozygosity (He) and Shannon's phenetic index (H) were estimated et al. [14].

Cluster analysis (Unweighted Pair Group Method using Arithmetic Averages) and principal coordinate analysis (PCoA) were performed to identify genetic variation patterns among the cashew genotypes using DarWin and NTSYSpc (version 2.2) software's respectively.

Results

Genetic polymorphism revealed by microsatellite markers

The SSR markers (Table 2) selected to analyze the genetic diversity of the cashew cultivars, displayed different characteristic profiles (Table 3).

Different numbers of polymorphic bands, percentage of polymorphism, Polymorphism Information Content (PIC), number of alleles and expected heterozygosis have been generated using the SSR markers (Table 3).

All microsatellite markers used were found to be polymorphic, in other words a loci polymorphic rate of 100% was observed. The number of polymorphic bands generated by each marker varied from 8 (with mAoR52) to 30 (with mAoR6) with a mean value of 18. The level of polymorphism ranged from 5.52% (mAoR52) to 20.68% (mAoR6). The discriminating power of each primer pair, estimated by the value of the PIC varied between 0.63 and 0.95 with an average rate of 0.81% for all SSRs analyzed.

Loci	Number of scored polymorphism	Percentage of polymorphism	PIC	He	N
mAOR3	18	12,41	0.82	0,42	2
mAOR6	30	20,68	0.63	0.50	3
mAOR7	25	17,24	0.71	0.48	2
mAOR11	13	8,96	0.90	0.33	2
mAOR17	13	8,96	0.90	0.33	2
mAOR42	21	14,48	0.77	0.45	2
mAOR48	17	11,72	0.83	0.40	2
mAOR52	8	5,52	0.95	0.23	2

Table 3: Number of scored polymorphic bands, percentage of polymorphism, estimated PIC, number of alleles (N) and expected heterozygosis (He) of eight SSR markers.

Among the 146 distinct scored bands (~3 bands/cultivars); 12% (n=17) were recorded for North -West cultivars, 47% (n=68) for Center's cultivars and 42% (n=61) for the North-East cultivars (Table 4).

Area	Number of cultivars	Number of Loci amplified	Pourcentage de polymorphisme	Shannon index
Central	28	68	46,89	0.065
North West	12	17	11,72	0.004
North East	20	61	42,07	0.052
Total	60	146	100	

Table 4: Genetic diversity of cultivars based on cashew growing geographical areas in Benin.

There were no specific bands belonging to cultivars of the same production area. The cultivars of Center and North-East showed a high polymorphism compared to those from the North Western region. The number of cultivars per zone had no effect on the

percentage of polymorphism. Shannon index varied between 0.004 and 0.065 with an average of 0.04 for all cultivars.

Genetic relationship and cluster analyses

Genetic relationships among cashew cultivars were determined by the Unweighted Pair Group Method with Arithmetic mean (UPGMA) method using the Nei distances [21]. The dendrogram profile of the *Anacardium occidantale L.* cultivars has been generated to elucidate the genetic diversity of the samples (Figure 2).

Figure 2: Dendrogram showing the genetic relationships between cultivars cashew by UPGMA analysis SAHN method using the coefficient of Nei (1972). Members of each genetic cluster are clearly depicted in Table 5.

Clusters	Sub-clusters	List of cultivars
I	A	E11-4; C18-1; O24-4; E2-4; E26-1; E15-1; E9-1; E34-2; E21-2; O17-1; C21-2; E32-2; C39-2; E6-1; C40-1; C22-2; C1-1; E39-4; E28-4; O12-4
I	B	E25-2; C28-2; C8-4; E31-2; C29-1; E13-4; C14-2; E5-1; E37-1; C24-1; E35-2; O32-1; C11-2; C31-4; C20-1
II		C25-1; C5-1; C9-3; C4-1; O16-4; C23-2; C16-1;C12-4; O27-1; O29-1; C10-1; C6-1
III		O20-1; O11-1; O14-3; E19-4; E18-4; O19-1; C43-3; E7-2; C26-2; C17-4; C33-3; O21-2; C3-2

Table 5: Classification of the cultivars by generated clusters.

At the dissimilarity level of 0.75, the cashew cultivars are grouped into three clusters: I, II and III (Table 5 and Figure 2). The Principal Coordinate Analysis (PCoA) of the cultivars (Figure 3) was profiled to further confirm the UPGMA-based dendrogram analysis (Figure 2). The first two axes of the PCoA show a great heterogeneity among morphotypes with almost 100% of the total variation, while axis 1 revealed 86.74% of the variation (Figure 3).

In agreement with the dendogram profile, the PCoA displayed three identical clusters with one cluster representing the cultivars from the central region of the country whereas the other two clusters displayed a heterogeneity of cultivars originated from all the three geographical locations of sample collection.

Figure 3: Principal Coordinate Analysis (PCoA) of cashew cultivated in Benin.

The cluster I consists of two sub-clusters (A and B) containing 35 cultivars and was 80% different from other clusters (II and III) (Figure 2). The 35 cultivars of cluster I are heterogenic and comprise cultivars from the three geographical collection sites. Sub-cluster A contains mainly 20 cultivars from the Northeast, that are agronomically and morphologically different from those of sub-cluster B. Cultivars in sub-cluster A produced small, round and conical apples with an oblique apex, small and round nuts and have a reduced flowering phenotype (Figure 4).

Figure 4: Phenotypic characterization of different genetic clusters of A. occidentale cultivars. The apple cashew (A, C, E, G) and nut (B, D, F) phenotypes of the genetic clusters are depicted. The nut phenotype of cluster III is not shown.

Sub-cluster B is composed of mainly 15 cultivars from the North-East and Central regions of the country. Sub-cluster B is 10% different from sub-cluster A. Sub-cluster B is made of very small apples and very small kidney- and round based-shaped nuts and have a reduced flowering phenotype and small inflorescence (Figure 4).

Cluster II is a homogenous population composed of 12 cultivars collected from the Central region of Benin, which is at least different from clusters I and III at the dissimilarity level of 0. 45. The cultivars of cluster II have medium level of flowering phenotype and produce very big apples with a round based, oblique and round apex and very large oblong nuts (Figure 4).

Cluster III was composed of thirteen cultivars collected from the three geographical collection regions. This group is as heterogeneous as cluster I. The cashew trees of this cluster are highly prolific with a very high flowering phenotype (i.e. produce a very high number of flowers especially hermaphrodite flowers). The plants in this group give cylindrical apples with round bases and two different colors (yellow and red) and without nervure (Figure 4). The nuts and apples produced by these plants were medium size (Figure 4).

Discussion

Polymorphism analysis

In this study, all SSR markers were polymorphic with a high discriminatory power (0.81 average) that allowed discrimination of morphotypes. The high level of the PIC values showed that the fragments generated in this study were very informative. The efficiency of the molecular marker technique depends on the level of polymorphism and discriminatory power among the set of accessions [22]. Our results corroborate those reported on cashew in Tanzania et al. [18], Nigeria [10] and on other species such as *A. humile* [23], *Dacryodes buettneri* et al. [24] and Sorghum [25].

The heterozygosity (average 0.40) obtained in this study was lower than that obtained by Croxford et al. [18] and Costa et al. [23] on cashew (0.46) grown in Tanzania and on A. humile (0.46) respectively. In contrast, the heterozygosity obtained in this study was higher than those reported by Todou et al. [24] on Dacryodes buettneri (0.35). However, our data showed a high genetic diversity in comparison to the results on cashew cultivated in other African countries such as Nigeria [10] and Tanzania [8]. The number of alleles are in agreement with previously reported Croxford AE et al. [18] with the exception of two loci (mAoR17 and mAoR42) that display lower allelic values. This might be explained by the different cashew cultivars analyzed in this study as well as the different numbers of accessions, because the numbers of alleles often correlate with the total number of samples analyzed.

Genetic diversity of cashew cultivars

The relatively low Shannon diversity index (0.04) obtained in this study suggests a low genetic diversity and differentiation of cashew cultivars in Benin. These results were in agreement with those obtained by Aliyu OM [10] in Nigeria, where the cashew cultivars studied had a high level of redundancy (low genetic diversity) using the same microsatellite markers. In addition, various studies using RAPD, ISSR and AFLP markers showed a low genetic diversity of cashew in Tanzania and India [8,26]. This high similarity of morphotypes can be explained by the selection criteria used by farmers to select seeds. According to our previous study on traditional knowledge of cashew production, two selection criteria, nuts size and productivity of cashew tree, have been used by farmers for the establishment of new plantations [4]. This practice of the seed choice could be the basis for the reduction in genetic diversity across different countries. It is therefore necessary to create a cashew germplasm collection in Benin to reduce this redundancy and create a database for conservation of the different cashew varieties in Benin. Considering the three regions of cashew production in Benin, we observed the highest diversity in the Central and the Northeast regions compared to the Northwest region of the country.

For a better understanding of the genetic diversity in cashew morphotypes, the genotyping data recorded for all the SSR markers were used to generate the UPGMA-based dendrogram (Figure 2) and principal component analysis (PCoA) (Figure 3). Both UPGMA-dendrogram and PCA displayed similar grouping of morphotypes, providing three groups of morphotypes that are genetically and morphologically different. The dendrogram shows a great similarity between plants of the same group. However, the grouping of morphotypes into different cluster reflects the genetic history, eco-geographic and agronomically affinity between the different clusters and morphotypes. In general, morphotypes were grouped on the basis of the apples and nuts characteristics, and flowering phenotype. Aliyu and Awopetu [5] used isozymes to obtain six clusters and 11 sub-clusters among the Nigerians cashew whereas India cashew were grouped into two clusters of which 1 consisted of a single cultivar and the second group consisted of 19 cultivars by using RAPD marker [2]. These results compared to ours show the robustness of our results and displays greater diversity of cashew cultivation in Benin.

Our results confirm those obtained using morphological markers [11]. In our previous study, morphological descriptors showed a grouping of the cashew cultivars in 4 clusters as a function of apple and nuts parameters and inflorescence [11]. It has been reported that the use of morphological traits is not always the best way to evaluate genetic distance since the degree of divergence between genotypes at the phenotypic level is not necessarily correlated with a similar degree of genetic difference [2]. Accordingly, the molecular markers provide a better coverage of the genome, resulting in a better estimate of relationships [2].

Cashew germplasm collection (core collection)

Endogenous knowledge of producers on *Anacardium occidentale L.* and genetic diversity revealed by SSR markers justify the establishment of the “core collection” of cashew in Benin. For the core collection establishment, the phenotypic and genotypic characteristics as well as endogenous knowledge of producers on these species should be taken into account. In our previous report, we described high endogenic knowledge and traditional-based descriptors for phenotypic classifications often used by cashew producers and also those developed by IBPGR on cashew cultivated in Benin [4,11]. Combining the above core collection descriptors with the results obtained in this study revealed a high genetic diversity of cashew cultivars in Benin. In order to conserve the diversity and improved the productivity of these cultivars, a core collection of cashew varieties in Benin is required. Similar strategies have been used by Archak et al. [26] to create a core collection of cashew cultivated in India.

Conclusion

In the present study, the SSR markers revealed the genetic relationships and diversity of cashew cultivated in Benin. This study provides useful information that can be used in a breeding program for genetic improvement and characterization of new varieties. In addition, the results of this study are relevant for developing management and conservation strategies for cashew genetic resources.

Additional Information and Declarations

Authors' contributions

SOK, KCS conceived the study. KCS, EWG, SOK wrote the paper. KCS performed the study. SOK, KCS, EWG analyzed, discussed and assessed the data. HA-S, LA, AS, AA, TK, LB-M contributed reagents/materials/analysis tools.

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