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Population Structure And Diversity Among Improved Cowpea Varieties From Senegal Based On Microsatellite Markers

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ABSTRACT

Cowpea (Vigna unguiculata) is used in West Africa for both human consumption and animal feeding. Despite its importance, the production of the crop is hampered by biotic and abiotic constraints. To overcome these constraints, the Senegalese Institute of Agricultural Research (ISRA) has set up a breeding program since 1960 leading to the release of productive varieties. Information of the genetic diversity for these varieties could improve the effectiveness of further breeding programs. The objectives of this study was to assess the genetic diversity and the population structure of 11 cowpea improved varieties from ISRA collection. Twelve (12) polymorphic microsatellite markers were used and the analysis of genotypic data showed a total of 39 alleles ranging from 2 (SSR6288, SSR6311 and SSR6827) to 6 (SSR6807) per locus. Polymorphism rate ranged from 8.33% (Mougne and Kelle) to 58.33% (Mouride) with an average of 31.06%. Intra varietal genetic diversity was very weak (0.012 to 0.091). The genetic similarity revealed that Melakh and Pakau varieties were genetically the closest while Bambey 21 and Sam were the most distant. These results are confirmed by the dendrogram, which also showed that Thieye, Leona, Kelle, Sam, Mougne and Yacine varieties have a higher genetic stability than Lisard, Melakh, Pakau, Mouride and Bambey 21. Eighty-seven percent (87%) of the total variability was attributed to inter varietal variability while 17% was due to intra varietal variation. These results provide a better knowledge of genetic resources of cowpea varieties released and held by ISRA and constitute important input for further cowpea breeding programs.

Keywords: Cowpea, Genetic diversity, Improved varieties, SSR markers, Senegal

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Introduction

To cope with the increase of populations in Sub-Sahelian Africa, estimated by the World Bank at 1.2 billion inhabitants in 2020, African agriculture will have to meet the needs of a rapidly increasing population, while reducing the number of people suffering from malnutrition. This requires a sustainable intensification of agricultural production, taking into account crops likely to generate substantial income and balance the populations food supply. Thus, the production of cowpea, the most important seed legume in the African tropical savannah zones and the second most important in Senegal, requires special attention. Cowpea (*Vigna unguiculata* (L.) Walp.) is part of the traditional cropping systems in Africa, where it is considered as a strategic crop because of its multiple uses as human food, animal feed, source of nitrogen for soil restoration and income for resource-poor farmers as well as small scale processors [1,2,3]. Cowpea plays a critical role in the lives of millions of people in Africa and other parts of the developing world where it is a major source of dietary protein that nutritionally complements staple low-protein cereal and tuber crops and is a valuable and dependable commodity that produces income for smallholder farmers (especially women who farm) and traders [4,5,6,7] In the dry savannah agro-ecologies of sub-Saharan Africa (SSA), Cowpea is an important grain legume grown especially because of its tolerance to drought [8].

Unfortunately, cowpea cultivation is neglected with an estimated annual world production of 7.2 million tons of dry seeds [9] with a low level of productivity in the Sahelian zone where it is a valuable staple food. In Senegal, its production still remains low, amounting to 152 753 tonnes from a cultivated area of 257 216 ha [10]. In order to improve its production, cowpea genetic improvement and production programs focus mainly on the combination of desirable agronomic traits. The knowledges of the genetic diversity within the existing collection can improve the effectiveness of these programs.

During the last decades, molecular variabilities present in plant material were determined using morphological, biochemical or DNA markers. However, the low availability of morphological markers, the lack of knowledge on genetic control, and the influence of environmental factors on phenotypic expression at different stages of development are major limitations for using these markers as tools in diversity studies [11]. It followed by the abandonment of biochemical markers, due to their limited number, sensitivity to environmental factors, and the developmental stage, in favor of markers based on DNA. Recently, molecular markers have provided new tools for analyzing genetic diversity but, even if certain such as RAPDs and AFLPs show a high level of polymorphism in genetically closely related genotypes [12,13,14,15], microsatellites are excellent genetic markers, locus-specific by their flanking sequences, highly polymorphic, reliable, reproducible, codominant, hypervariable, informative and are very abundant in the genome [16]. Thus, with the wide application of the molecular methods, breeding programs have remarkably accelerated the creation of new productive varieties.

Genetic diversity research being a crucial premise for breeding [17], efforts have been made on cowpea to assess the genetic diversity of the global collection from Africa and other localities in America and Asia where it is cultivated [18,17,19]. In Senegal, despite studies by [13,20,21], who examined pure varieties and lines of cowpea, the genetic diversity and phylogenetic relationships of cowpea available in the ISRA collection remain poorly understood. The purpose of this study is to assess the genetic diversity and the population structure of 11 cowpea improved varieties from Senegal. More specifically, it is to evaluate for the first time the genetic diversity of 5 new varieties released in 2015 with 6 others from the ISRA collection.

Materials and methods

Plant materials

Eleven (11) cowpea improved varieties (Melakh, Mougne, Yacine, Mouride, Bambey 21, Pakau, Lisard, Thieye, Leona, Kelle and Sam) (Table 1) provided by the senegalese national breeding program were used in this study. These varieties were developed by the CNRA/ISRA cowpea breeding team at Bambey during the five last decades. Among these varieties, five (Lisard, Thieye, Leona, Kelle and Sam) have recently been released [22].

With regard to the number of samples, 24 samples were taken per variety for the six old varieties, for a total of 144 samples (due to the fact that previous molecular genetic studies have been done on these old varieties). For the 5 new varieties, 96 samples per variety were taken; giving a total of 480 samples. For these 5 new varieties, the present study is the first molecular genetic assessment. This justify the use of 96 samples per variety (instead 24 samples per variety like in the old varieties) in order to assess the genetic stability of these new varieties.

Thus, a total of 624 samples were used in this study.

Genomic DNA extraction and amplification

DNA extraction was carried out using fresh young leaves from 21-day-old seedlings using the modified MATAB (Mixed Alkyl Trimethyl Ammonium Bromide) extraction method [23,24]. The extracted DNA was quantified on 1% agarose (Promega Corporation) gel by visual estimation based on comparison with the bands of Smart Ladder.

Fourteen (14) polymorphic microsatellite markers (Table 2) were used for DNA amplification. A reaction volume of 10 µl, consisting of 5 µl of DNA (5 ng/µl) and 5 µl of a PCR solution was used. The PCR solution contained 1.1 µl of 10X buffer, 1.1 µl of dNTPs (200 µM), 0.11 µl of MgCl2 (0.5 mM), 0.09 µl of each primer (0.1 µM), 0.09 µl of IRdye (0.1 µM), 0.275 µl of Taq polymerase (1U). The PCR was carried out in a 96 block thermal cycler (MWG AG biotech) according to the following general conditions: an initial denaturation step of 4 min at 94°C, followed by 35 cycles of 60 s at 94°C, 1 min at 50 or 55°C (depending on the primers), 1 min 15 s at 72°C and a final extension for 7 min at 72°C.

Table 1 : Agromorphological characteristics of the 11 varieties used in this study [20,22]

Varieties	Pedigree	Date of approval	Growth habit	Flowers	Seeds color	One hundred seed weight (g)	Yield
Melakh	IS86-292xIT83s-742-13	1997	Prostrate	White	White, brown-eyed	21	1.8
Pakau	Mouridex58-77	2011	Semi-erect	White	Brown, white-eyed	20	3.5
Mougne	58-74xPout	1974	Prostrate	Bicolor white	Cream punctuated with gray	15	0.9-1.4
Yacine	MelakhxNdiagaAw	2005	Erect	White	Brown, white-eyed	23	2.5
Mouride	5857xIT81D-1137	1995	Semi-erect	Bicolor	Cream, beige-eyed	18	2.277
B 21	5/8 de 58-40+1/4 de 66-74+S	1974	Erect	White	White	17	1.5-2
Lisard	MelakhxMonteiro	2015	Erect	Conclore, White	White, light brown-eyed	27.35	3.1
Thieye	MelakhxMonteiro	2015	Erect	Conclore, White	White, light brown-eyed	27.35	3
Leona	MelakhxMonteiro	2015	Semi-Prostrate	Conclore, White	White, light brown-eyed	25.1	3
Kelle	MelakhxMonteiro	2015	Semi-erect	Conclore, White	White, light brown-eyed	26.7	2.9
Sam	MelakhxMonteiro	2015	Erect	Conclore, White	White, brown-eyed	23.93	3.3

Table 2: List of primers used in this study

Primer codes	Sequences (5'-3')	Tm◦C
SSR6215-MF	CACGACGTTGTAAAACGACGCTTCCCCGCTAGAATCTTT	81,7
SSR5215-R	GGTGCCAATGGATCAGGTAA	64,6
SSR6288-MF	CACGACGTTGTAAAACGACGATGTTGTAGCAGGCTAATTGGA	81,7
SSR6288-R	TGGCCAATTGTCCTAAGTTGA	64,2
SSR6218-MF	CACGACGTTGTAAAACGACGTGGAAGGAATGGGTCCAG	83,7
SSR6218-R	AGGAAATTTGCATTCCCTTGT	63,4
SSR6769-MF	CACGACGTTGTAAAACGACGATCATTGTAATCCTATTGACTAC	76,9
SSR6769R	GAACTTGGAAACAATATCTACGG	60,3
SSR6311-MF	CACGACGTTGTAAAACGACATGCCATTGTTGAGTTGCTTT	81,5
SSR6311R	AGGATGTTGTAGCAGGCTAATTG	63,1
SSR6807-MF	CACGACGTTGTAAAACGACGAACTATTATACAATCATGCACGA	79,2
SSR6807-R	GTAGCTTACTTCAATGATTAG	50,1
SSR6289-MF	CACGACGTTGTAAAACGACCCCCCAAAGTTGATGAACAC	82,5
SSR6289-R	TTGATGGAGTTCGCATCTTCT	63,7
SSR6827-MF	CACGACGTTGTAAAACGACCTCTCACATGCAATCCTAAATGGC	82,9
SSR6827-R	CTACGATAATGAGGATAACCATC	58,1
SSR6304-MF	CACGACGTTGTAAAACGACCTCTCACATGCAATCCTAAATGGC	82,9
SSR6304-R	CTACGATAATGAGGATAACCATC	58,1
SSR6323-MF	CACGACGTTGTAAAACGACCAAAGGGTCATCAGGATTGG	82,6
SSR6323-R	TTTAAGCAGCCAAGCAGTTGT	63,7
MA113-MF	CACGACGTTGTAAAACGACTCGCACACAGATCCAACATT	81,9
MA113-R	CCTTATTTCTGGTGGGAGCA	63,9
MA118-MF	CACGACGTTGTAAAACGACCACCACTCTCCTGCACTCAA	83,4
MA118-R	GGTTGGGCAAAGAGGAACTA	64,8
MS120-MF	CACGACGTTGTAAAACGACTTTCTAGGCAGTGAAGATAATCA	77,7
MS120-R	AAACAAAATACCAACTACCA	53,7
Y87-MF	CACGACGTTGTAAAACGACCGCCAAAACCCCGATAACC	84
Y87-R	GCCCCCTCCGACTG	43

Electrophoresis on LI-COR DNA Analyzer system

DNA electrophoresis and visualization was performed on 6.5% denaturing polyacrylamide gel using the LICOR 4300 sequencer (LICOR Inc., NE, USA). The LICOR system also allows the mixing of PCR products of two markers prior labeled with IRdye primers (IRdye 700 and

IRdye 800). Before loading, the multiplexed PCR products and a size marker were denatured at 94°C for 3 min. Ninety-six (96) samples were simultaneously loaded into the acrylamide gel using a membrane comb. The fragments, prior marked with IRdye during amplification, emit fluorescence when excited by laser diodes at two different wavelengths (682 and 782 nm). An infrared camera detects the signals and the images are automatically recorded and downloaded for analysis. Allele sizes were estimated by comparing with different bands of the size marker (ladder produced by CIRAD) [25].

Data Analysis

All the images of the gel profiles have been processed with XnView 3.13 software and imported into the Jelly 0.1 application for reading. The alleles size was estimated using the different bands of Smart ladder. The alleles are coded using numbers (1, 2, 3, 4, 5 and 6). Missing data were coded 0. Excel sheet was used for the coding. Genetic parameters such as number of alleles per locus, percentage of polymorphic loci, heterozygosity, genetic similarity and analysis of molecular variance were determined using GenAlex 6.4 software [26].

Population structure was estimated using STRUCTURE software [27] and DARwin [28]. STRUCTURE analysis was performed with K ancestral populations ranging from 1 to 10. We used 200,000 iterations and a burn-in period of 20,000; 10 runs for each K value were performed. Outputs were summarized using Structure Harvester [29]. We evaluated the number of population K based on Evanno's method [30]. For ancestry analysis (q), we used the simulation with the highest log probability. We classified individuals based on an ancestry coefficient of 0.7 or higher. Then a genetic dissimilarity dendrogram was carrying out with Darwin software.

Results

DNA diversity revealated by SSR markers

Among the 14 microsatellites used, 12 gave clear amplification of DNA of all the varieties. The migration profil of marker SSR6769 was difficult to analyse because of the high size of alleles that didn't allows good separation. Marker SSR6218, amplified the six oldest varieties (Melakh, Pakau, Mougne, Yacine, Mouride and Bambey 21) but didn't amplified the five new varieties (Lisard, Thieye, Leona, Kelle and Sam). Therefore, these two loci were not considered in the data analyses.

The analysis carried out based on the 12 polymorphic loci data, allowed the detection of 39 alleles with a mean value of 3.25 alleles per marker. The number of alleles per locus (N.al) ranged from 2 (SSR6288, SSR6311 and SSR6827) to 6 (SSR6807).

The average of the observed heterozygosity (mHo) is zero for all loci except MA118, MS120 and SSR6807 that show a very low mHo (0.008, 0.011 and 0.007 respectively). The expected heterozygosity is zero for two loci and varied between 0.029 and 0.156 for the others loci (Table 3).

Table 3: Number of alleles and mean heterozygosity per locus

Loci	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12
N. al	3	3	3	4	2	4	3	2	3	6	2	4
mHe	0.042	0.029	0.066	0	0.048	0.043	0	0.043	0.013	0.156	0.014	0.053
mHo	0	0.008	0.011	0	0	0	0	0	0	0.007	0	0

Legend: N. al = Number of alleles; mHe = expected heterozygosity means ; mHo = observed heterozygosity means ; M1 = MA113 ; M2 = MA118 ; M3 = MA120 ; M4 = SSR6215 ; M5 = SSR6288 ; M6 = SSR6289 ; M7 = SSR6304 ; M8 = SSR6311 ; M9 = SSR6323 ; M10 = SSR6807 ; M11 = SSR6827 ; M12 = Y87

Genetic diversity of varieties

Genetic diversity was analyzed among the different varieties based on the polymorphism data from the 12 SSRs. The percentage of polymorphic loci per variety ranged from 8.33% (Mougne, Kelle) to 58.33% (Mouride) with an average of 31.06% (Table 4).

The observed heterozygosity mean (mHo) is zero for five varieties (Pakau, Mougne, Yacine, Thieye, and Leona) and very low for the six others (Melakh, Mouride, Lisard, Kelle, and Sam where it varied between 0.001 (recorded in Lisard) to 0.042 (recorded in Bambey 21). The expected heterozygosity mean for each variety is very low and ranged from 0.012 (observed in Leona) to 0.091 (observed in Bambey 21) (Table 4).

Genetic similarity between varieties

Table 5 shows the genetic similarity between varieties. The values of genetic similarity are ranged from 0.105 to 0.998. The highest similarity value is observed between Melakh and Pakau while Sam and Bambey 21 (B21) have the lowest similarity value. Bambey 21 has the lowest genetic similarity with the others varieties (between 0.105 and 0.294). The varieties Lisard, Thieye, Leona, Kelle and Sam showed high genetic similarities (>62%).

Table 4: Percentages of polymorphic loci and mean heterozygosity per varieties

Varieties	Melakh	Pakau	Mougne	Yacine	Mouride	B21	Lisard	Thieye	Leona	Kelle	Sam
%P	50	50	8.33	16.67	58.33	41.67	50	25	16.67	8.33	16.67
mHo	0.003	0	0	0	0.003	0.042	0.001	0	0	0.005	0.001
mHe	0.062	0.071	0.013	0.019	0.069	0.091	0.063	0.042	0.012	0.031	0.016

Legend: B21 = Bambey 21 ; %P = percentages of polymorphism; mHe = expected heterozygosity means ; mHo = observed heterozygosity means

Table 5: Genetic similarity between cowpea varieties

Melakh	Pakau	Mougne	Yacine	Mouride	B21	Lisard	Thieye	Leona	Kelle	Sam	
1.000											**Melakh**
0.998	1.000										**Pakau**
0.507	0.503	1.000									**Mougne**
0.744	0.742	0.423	1.000								**Yacine**
0.359	0.373	0.187	0.516	1.000							**Mouride**
0.361	0.373	0.271	0.457	0.650	1.000						**B21**
0.754	0.755	0.433	0.720	0.305	0.207	1.000					**Lisard**
0.745	0.746	0.430	0.620	0.298	0.285	0.912	1.000				**Thieye**
0.826	0.825	0.371	0.645	0.297	0.211	0.906	0.906	1.000			**Leona**
0.688	0.688	0.269	0.562	0.485	0.294	0.775	0.776	0.851	1.000		Kelle
0.595	0.596	0.338	0.583	0.181	0.105	0.854	0.769	0.740	0.625	1.000	Sam

Legend : B21 = Bambey 21

Molecular variance analysis

The results of the molecular variance analysis (AMOVA) revealed that 87% of the total diversity is at the inter-varietal level (table 6).

Genetic structuring of the 11 cowpea varieties

The structuring of 11 cowpea varieties distinguishes ISRA collection resources into nine (9) genetic groups (Fig 1a). Groups I, III, IV, V, VI, VII, IX, are homogeneous and include individuals from Thieye, Lisard, Yacine, Leona, Kelle, Sam, and Mougne varieties respectively. Groups II and XIII are the most heterogeneous, each containing two varieties. Group II consists of Melakh and Pakau and group XIII those of Bambey 21 and Mouride varieties (Fig 1b).

Sixteen individuals (10 of Lisard, 2 of Pakau, 2 of Thieye 1 of Mouride and 1 of Melakh) are hybridized with a membership coefficient to a genetic group lower than 0.7

Table 6. Analysis of molecular variance (AMOVA) of the 11 cowpea varieties. Within Pops = within varieties; among Pops = among varieties

Source	df	SS	MS	Est. Var.	%
Among Pops	10	4433.903	443.390	8.118	87%
Within Pops	613	772.677	1.260	1.260	13%
Total	623	5206.580			

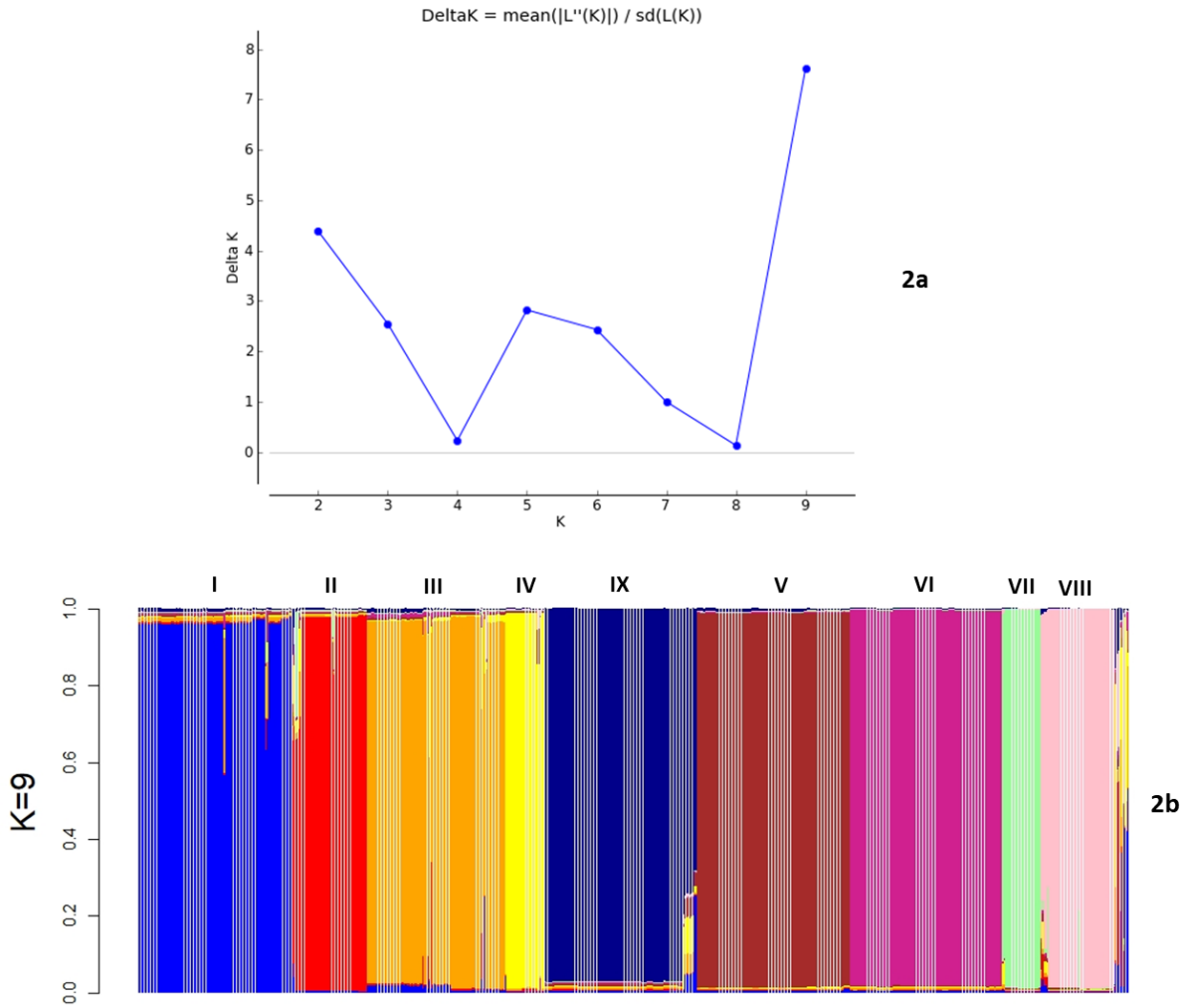

Fig 1: Population structure analysis for cowpea improved varieties from ISRA collection. 2a ΔK criterion according to the calculation method by Evanno *et al.* (2005). 2b Structure of 11 cowpea improved varieties from ISRA based on 12 SSR markers

The structuring with Darwin software shows a dendrogram with four large genetic groups and several subgroups. The first group (a) consists of 4 varieties (Melakh, Pakau, Lisard and Leona). The second group (b) includes of all individuals of Kelle variety and one individual of Sam variety. The third (c), the largest group, includes of 5 varieties (Bambey 21, Mouride, Mougne, Yacine, and Thieye). This group contains all the former improved varieties (except Melakh and Pakau) in four different sub-groups very homogeneous. Regarding the fourth group (d), it contains only individuals of Sam with 93 % bootstrap value. Individuals of Thieye and Leona varieties are distributed in groups a and c, and only one individual of the Melakh variety was found in group c (Fig 2).

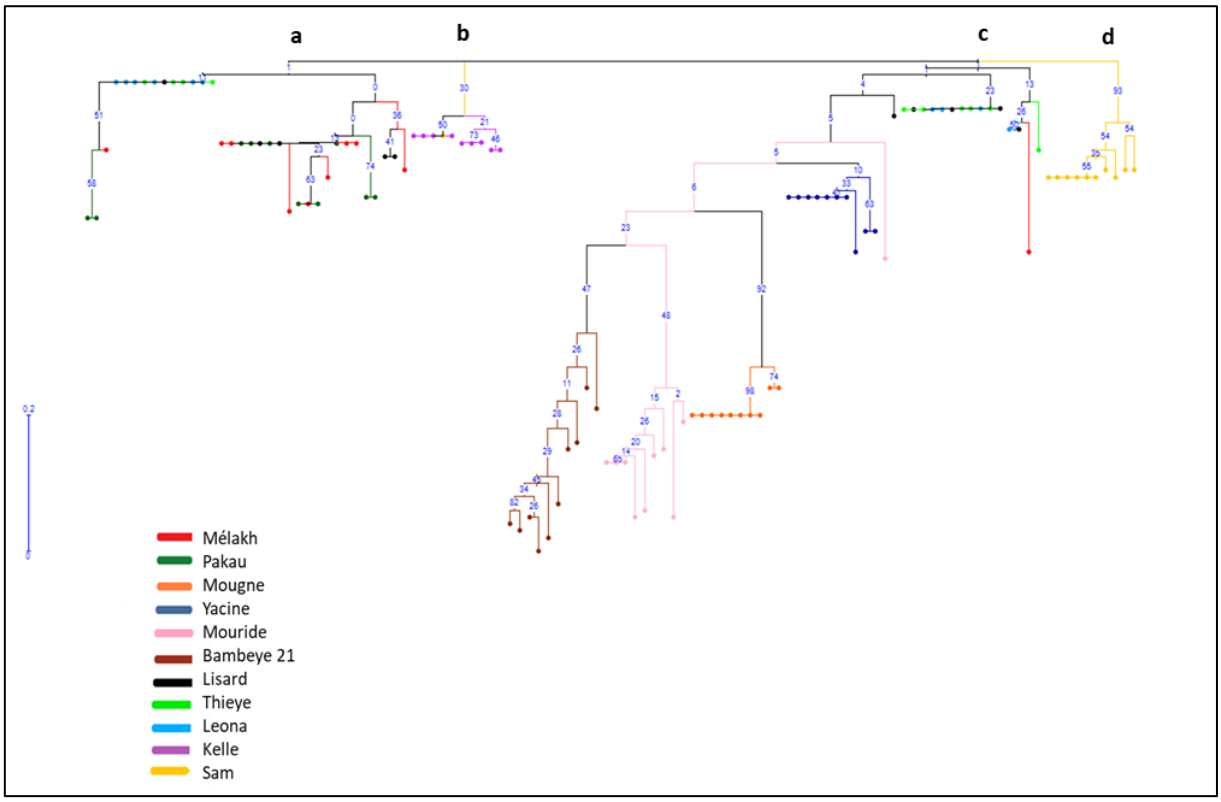

Fig 2 : Genetic dissimilarity dendrogram of the 11 cowpea varieties

Discusion

A low genetic diversity has been recorded in this study. The intensification of human action has led to a significant reduction of genetic especially in improved varieties [31]. Indeed, the marker SSR6218 which amplified only the six old varieties could be associated with a trait absent in the five new ones. Therefore, assessment of the genetic diversity of ISRA's cowpea collection is necessary for its management, conservation and for further breeding programs. The number of alleles per marker detected in this study, ranged from 2 to 6. These values are relatively high in comparison with results of [20] that found 2 to 3 alleles per marker by gentotyping seven varieties from the ISRA collection. However, this number is lower than those found by [13] that recorded 1 to 9 alleles per marker and [21] that found 1 to 16 alleles per marker. These two latter works were carried out on 11 and 22 traditional and selected varieties respectively from Senegal.Very recently, [25] obtained allele numbers per marker ranging from 2 to 15 in their genetic diversity studies on a panel of 671 traditional local cowpea accessions. These differences noted between the number of alleles found in cowpea from Senegal could be explained by the types (traditional and improved) and number of varieties, the technique used

during DNA separation or the number and the sequences of markers used in each study.

The total heterozygosity obtained in this study is very low, suggesting a narrow genetic diversity within the varieties. This seems logical because as well purified improved varieties should be present no heterozygotes. The absence or low presence of the observed heterozygotes can also be explained by cowpea reproduction system which is characterized by a high degree of autogamy. Autogamy prevents a random union of gametes and individuals, resulting in the decrease of heterozygotes [32]. The genetic diversity found is lower than that obtained by [32,25] who studied on traditional cowpea varieties. [21] also found high values of heterozygotie on local varieties and inbred lines collected across Senegal. The low genetic diversity recorded in this work reflects lower genetic variability in improved varieties compared to that found on local traditional varieties.

Genetic analysis showed that genetic similarities between varieties are highly variable (ranging from 0.106 to 0.998). These values suggest that there are varieties that are very close genetically and others that are very distant. The highest similarity value observed between Melakh and Pakau means that they are genetically the closest. These two varieties also have morphologically very similar seeds. Lisard, Thieye, Leona, Kelle and Sam varieties were obtained by crossing the variety Melakh and Monteiro (Brazilian variety). This explains the high genetic similarity between these varieties and the parent Melakh. Bambey 21 and Sam are genetically more distant with the lowest similarity value (0.106). These two varieties being the most contrasted could be used in future breeding programs. A previous genetic variability recorded on seven varieties (Melakh, Mouride, 906, Ndiaga-Aw, 961, Bambey 21, and Isra-819) from the ISRA collection distinguished Bambey 21 and Mouride from the others [20]. Indeed, these results are confirmed by the analysis of molecular variance which revealed a higher genetic differentiation (87%) between varieties than within them (13%). This intra-varietal diversity, although low, is a hindrance for breeding programs that requires stable genetic material. Nevertheless, the percentage of intra-varietal diversity obtained is significantly better than that of [32] who found 38% on traditional varieties.

AMOVA results are confirmed by those of the dissimilarity dendrogram of the 11 cowpea varieties. Mougne, Yacine, Mouride, B21, Pakau and Kelle varieties have all their individuals in the same large genetic group. Melakh, Sam and Lisard, belong to the same group even if few individuals of these varieties were found in another group. However, Thieye and Leona have intertwined and form polytomies in two different genetic groups. These results would suppose a possible mixture of seeds between these varieties hypothesis that seems to be confirmed by a high similarity in seeds morphology. Mougne, Mouride and Bambey 21, varieties were found in the same group and Melakh in another, while [13], by combining the RAPD and SSRs techniques, found a single group for these four varieties. The grouping of Melakh and Pakau in the same genetic group suggests that they probably share a common parent. Further tests on these five new varieties could help to confirme results obtained in this study.

Conclusion

Genetic analysis conducted in this study revealed low genetic diversity within the cowpea improved varieties developped by ISRA and a high variability between them. It shows a high genetic similarity between Melakh, Pakau, Lisard, Thieye, Leona, Kelle and Sam varieties and moderate similarity with Yacine. Bambey 21 and Sam varieties are genetically the most distant. The analysis of molecular variance confirmed the existence of high inter-varietal genetic diversity and low intra-varietal diversity.

These results are importants inputs for better exploitations of ISRA cowpea collections and could help in the choice of the most genetically

contrasting and stable parents for breeding programs.

Conflict of interest

The authors have no conflict of interest to declare.

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