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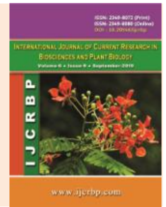

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Genetic diversity of *Hibiscus cannabinus* L. of Burkina Faso using SSRs markers

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ABSTRACT

This study is aimed at a better knowledge of the genetic diversity of *H. cannabinus*, a leafy vegetable which is very consumed in Burkina Faso. For this, a molecular characterization using SSRs microsatellite markers of 70 accessions collected in the three climatic zones of the country was carried out. The analysis shows significant genetic diversity among accessions, structured into three genetic groups. Indeed, 17 of the 20 markers tested were polymorphic. Expected unbiased heterozygosity ranged from 0.131 for marker MJM566 to 0.435 for marker MJM606 with an average of 0.309 for all primers. The Shannon diversity index (I) ranges from 1.552 for the primer MJM566 to 5.008 for the primer MJM606 with an average of 3.73 for all the primers tested. The organization of this genetic diversity is strongly influenced by the genetic group factor with a value of the differentiation index Fst of 0.324.

Introduction

Hibiscus cannabinus L., commonly known as "Kenaf", is a woody plant belonging to the family Malvaceae (Webber et al., 2002). It is a diploid species whose chromosome formulas 2n = 36, 2n = 72 are the most encountered. It is used both in human food as a vegetable and in animal feed as fodder. The young leaves, rich in digestible proteins, can be consumed green or after desiccation. They are palatable and can serve as a source of protein for ruminants and carotene for layers. The cultivation of *Hibiscus cannabinus* is gaining momentum in several localities in Burkina Faso (Millogo-Rasolodimby, 2001), where it is increasingly being grown in the dry season to meet growing consumer demand. Currently, it occupies an important place in peri-urban agriculture and provides important income to the various links in the value chain, thus contributing to the fight against poverty, especially that of women. Thus, leaves and seeds are marketed in local markets in several provinces of the country during the lean season between May and June (Millogo-Rasolodimby, 2001). Given its proven socio-economic and nutritional importance, estimating

its diversity for genetic improvement is still not a priority in Burkina Faso. Hence the interest of this study, which aims to evaluate the genetic diversity of a collection of *H. cannabinus* from seven provinces of Burkina Faso using SSRs microsatellite markers. More specifically, it aims to determine the level of genetic diversity of *H. cannabinus* from Burkina Faso; to evaluate the distribution of allelic frequencies between geographical regions and to define the potential genetic groups of cultivated *H. cannabinus*.

Materials and methods

Materials

Plant material: The plant material consists of seventy (70) accessions of *H. cannabinus*, collected in the three climatic zones of Burkina Faso, of which one (01) accession collected in the Sahelian zone, 32 collected in the Sudanian zone and 37 from the Sudano-Sahelian zone. An earlier study of agromorphological characterization structured 70 accessions into six (06) morphotypes based on stem color. These were green morphotype, purple morphotype, violet morphotype, black morphotype, red morphotype, and spotted green morphotype.

Microsatellite markers: Twenty (20) SSRs microsatellite markers were used to study the genetic diversity of *H. cannabinus*. These markers are the same, already used by (Satya et al., 2013) (Table 1).

Methods

Extraction of genomic DNA

The total genomic DNA of each of the 70 accessions was extracted from 0.2 g of fresh leaf according to the CTAB method described by Agbangla et al. (2002). At the end of the extraction, the DNA ball was taken up in a Tris-EDTA (TE) buffer solution. After verification of the quality of the DNA on agarose gel (2%), the tubes were stored in a freezer at -20°C. until the PCRs were carried out.

Table 1. Characteristics of SSR markers tested.

SSR	Primer sequence Forward	Primer sequence Reverse	Tm°C
MJM 06	ACGTTTAGCAACTGATATTGG	ACTTACAGCGGTTACATCATT	43.4
MJM 211	ACGACAATCAATACGACAATC	ATTCAGGCTTGATAACAGTGA	43.4
MJM 217	AATTACAAACTGGAGGTGGTC	TCAAATCCAAGCACCCATAAA	44.35
MJM 464	GGTATTACGGTGCATCAGCAT	CAGCAGCAACAGGTGTCATAA	47.3
MJM 475	TTGCTGCTTGATACAACTGGA	TACGAAACGACAAAGTCCACC	46.3
MJM 609	AATGGAATGGAGCTAACATCT	AGAATTTGCGAAGTGGGCTAT	44.35
MJM 563	CTTGGTTGTGGTGGTTGAACT	AAACCCACCATAGTTGTGTGC	47.3
MJM 618	CGTTATCAAGCAAATCCAACC	CATCTGGTGACTGCTTCGTCT	47.25
MJM 623	TTCTGCAGTTGTCTCCCTGTT	ACGAGAAGACACAGTGGTGCT	48.25
MJM 566	CGCCAGAGAAGCAAATGTAAC	TAGAGCTCACCAGAGACTGCC	49.25
MJM 606	GGTACTGGTGCATGCTGATTT	TTCTGTGGAACCTGAGCATCT	47.3
MJM 591	TGTCACTTGCCTATGATCGTG	AAACAACACCATGAACAGCAT	45.35
MJM 317	GGAAGGAGAAGAAGAAGAAA	CACCAATATTACGGGATAACA	49.7
MJM 467	CATGAATTGAGTGAGCATCCA	ATCTTCAAGCCCAAATATGCC	45.3
MJM 469	TGATTAGGCCTATTGAAGCG	AACTTGCCTAGGAGCTTGAGG	53.6
MJM 471	CCCACACACATACACACACAC	GATGATAATGACGACGAACCC	48.25
MJM 462	AAAGTGCAAATGAATTGGAGG	TCTTTGGCGTTTATGATGGTC	44.35
MJM 215	CCAGCTTCTCAAATCGTTTAT	CTTTAACTTGTTCGTCTGCAT	43.4
MJM 472	CCATTCGTAGCATTAAAGTTTGC	GATTGTGTGCAAACACGAGAG	46.95
MJM 486	TCGTCTGTACCTCTCTTCATGC	CCTCCCTGGACCATATCAAAT	48.45

PCR amplification

The PCR reactions were carried out in a final volume of 20 µl containing 1 µl of the 3'primer (forward primer), 1 µl of the 5' primer (reverse primer), 9 µl of ultra-pure water, 5 µl of genomic DNA of concentration 5 ng / µl and 4 µl of premix PCR composed of 1U of Taq polymerase, 250 µM Tris-HCL, 10 mM KCl, 1.5 mM MgCl 2. After homogenization, the reaction mixture was then placed in a thermal cycler for PCR amplification. This amplification was performed according to a

program consisting of an initial DNA denaturation phase at 95°C (5 min), followed by a series of 35 cycles. Each cycle consists of a denaturation phase at 95°C for 45 s, hybridization at TM (°C) for 1 min, extension at 72°C for 1 min, final elongation at 72°C for 10 min and finally cooling to 4°C.

Electrophoretic migration and reading of the bands

The amplification products were then subjected to electrophoresis on a 2% agarose gel prepared with a 1 X TBE solution. Thus, 8 µl of each PCR product was deposited in each well of the agarose gel at 2%. The deposits were made in the presence of a ladder marker consisting of 13 steps, ranging from 50 to 650bp. The migration was made at 80 V for 1 h 30 min in 0.5x Tris Borate EDTA buffer (TBE). At the end of the migration, a solution of 5% Ethidium Bromide (BET) was used as developer. Tape playback was done using a DI-01-220 model transilluminator surmounted by a 10 mega pixel camera. These bands were identified on the basis of their position on the gel. A binary coding was used for this, 1 was noted in case of presence and 0 in case of absence of band for each individual and for each primer tested.

Statistical analyzes of molecular data

The markers that gave fluorescent bands were retained for statistical analyzes. Genetic diversity within accessions has been analyzed at two levels: intra-population variability and inter-population variability. To do this, the genetic parameters were calculated using GenALEx software to evaluate the level of diversity of the entire collection. The organization and structuring of the genetic diversity of *H. cannabinus* was carried out using the DARWIN software through the "Neighbor-Joining" classification. Genetix software was used to calculate Nei distances between the different genetic groups obtained. Finally, the Genstat software was used for one-way analysis of variance, in order to verify the significance of the influence of the"morphotypes" and "genetic groups" factors on the structuring of the different genetic groups formed.

Results

Level of diversity of SSR markers tested

Seventeen (17) of the twenty (20) SSR markers used tested were polymorphic to three (MJM215, MJM472, MJM486) that were monomorphic (Table 2). Polymorphic markers revealed a total number of 87 alleles ranging in size from 50 to 650 bp. The number of alleles per locus ranged from 3 for primers MJM462, MJM606, MJM618 to 7 for MJM06, MJM217, MJM475, an average of 5 per polymorphic primer. The effective number of alleles (Ae) ranged from 2.45 (MJM469) to 5.75 (MJM217) with an average of 3.87 while expected unbiased heterozygosity ranged from 0.131 (MJM566) to 0.435 (MJM606) with an average of 0.309. All 17 primers have expected nonzero heterozygosity values. The Shannon diversity index (I) ranges from 1.552 for the primer MJM566 to 5.008 for the primer MJM606 with an average of 3.73 for all the primers tested. The polymorphism information (PIC) potential ranged from 0.131 (MJM566) to 0.438 (MJM566) with an average of 0.311. The size of alleles varied with a polymorphic (P) loci rate of 96.08% for all 17 polymorphic primers. The Fig. 1 is a migration profile obtained with the MJM475 marker for 20 individuals.

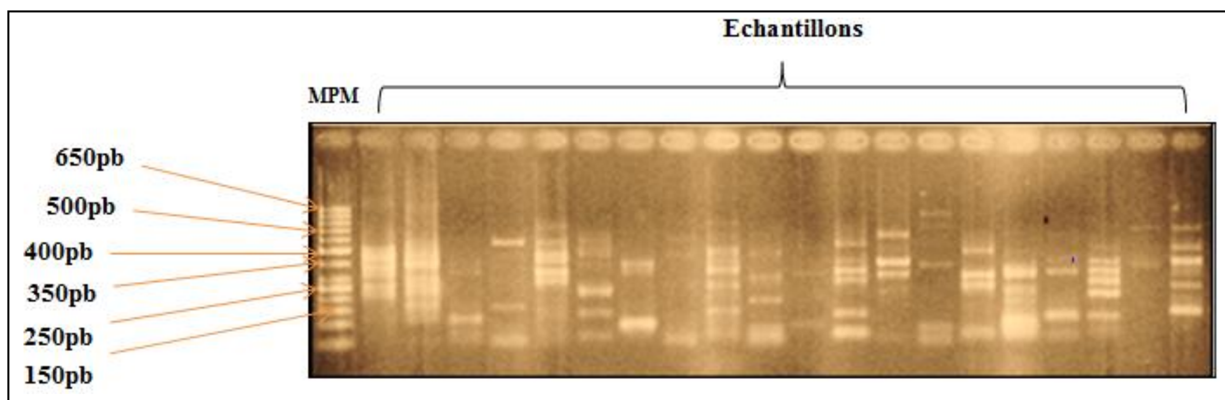

Fig. 1: Migration profile obtained with the marker MJM475.

Table 2. Level of genetic diversity of the 17 primers tested.

Locus	A^t	Ae	He	I	PIC	P (%)
MJM06	7	5.33	0.221	2.896	0.223	96.08
MJM211	5	3.45	0.300	3.752	0.302	96.08
MJM217	7	5.75	0.417	4.824	0.420	96.08
MJM317	6	4.77	0.410	4.712	0.413	96.08
MJM462	3	2.47	0.270	3.216	0.272	96.08
MJM464	6	4.48	0.301	3.704	0.303	96.08
MJM467	4	2.55	0.327	3.928	0.329	96.08
MJM469	4	2.45	0.286	3.520	0.288	96.08
MJM471	6	4.46	0.281	3.504	0.283	96.08
MJM475	7	5.57	0.342	4.144	0.345	96.08
MJM563	5	3.33	0.215	2.736	0.216	96.08
MJM566	5	3.21	0.131	1.552	0.131	96.08
MJM591	4	2.58	0.339	4.080	0.342	96.08
MJM606	3	2.78	0.435	5.008	0.438	96.08
MJM609	6	4.29	0.196	2.576	0.198	96.08
MJM618	3	2.77	0.430	4.952	0.433	96.08
MJM623	6	4.59	0.359	4.312	0.361	96.08
Average	5	3.87	0.309	3.730	0.311	96.08

Legend: At: total number of alleles, Ae: number of efficient alleles, He: expected heterozygosity, PIC: Polymorphism Information Content, I: Shannon diversity index, P: polymorphic loci rate.

Genetic diversity of the *Hibiscus cannabinus* collection "between morphotypes"

Morphotypes analysis shows intra-morphotype diversity (Table 3) and differentiation between morphotypes (Table 4). Thus, with an effective number of alleles of 3.75; 10 private alleles, a Shannon diversity index of 2,730; an expected heterozygosity of 0.300, a polymorphic information potential of 0.308 and a polymorphic loci rate of 92.16%, the green morphotype has the highest genetic diversity with the highest genetic parameters. The longest Nei minimum distance of 0.276 is observed between the purple and black morphotypes while the greatest Fst (0.2258) is obtained between the red and black morphotypes. The lowest value of Nei's minimum distance (0.059) is observed between the purple and the purple morphotype. The lowest differentiation index Fst (-0.0622) is observed between the purple morphotype and the black morphotype.

Table 3. Distribution of genetic diversity by six morphotypes.

Morphotype	A_e	A^p	I	He	PIC	P (%)
Green	3.75	10	2.730	0.300	0.308	92.16
Purple	3.32	1	1.872	0.202	0.216	65.69
Violet	3.53	0	2.082	0.237	0.296	58.82
Red	3.02	0	1.098	0.124	0.165	31.37
S.G.	3.18	0	1.526	0.153	0.229	34.31
Black	2.81	0	0.704	0.064	0.127	12.75
Average	2.80	2	1.668	0.180	0.223	49.18

Legend: Ae: effective number of alleles, Ap: number of private alleles, I: Shannon diversity index, He: expected heterozygosity, PIC: polymorphism information content, P (%): polymorphic loci rate, S.G.: Spotted Green.

Table 4. Inter-morphotypes genetic differentiation.

Morphotypes	Minimal Nei distance						Differentiation index Fst					
	Green	Purple	Violet	Red	S.G.	Black	Green	Purple	Violet	Red	V-T	Noir
Green	0.000						0.0000					
Purple	0.113	0.000					0.0183	0.0000				
Violet	0.099	0.059	0.000				0.0348	0.0168	0.0000			
Red	0.142	0.165	0.189	0.000			0.0171	0.0592	-0.0074	0.0000		
S.G.	0.176	0.174	0.186	0.127	0.000		0.0370	-0.0175	0.0497	0.1406*	0.0000	
Black	0.205	0.276	0.236	0.086	0.155	0.000	0.1005*	-0.0622	0.0932	0.2258*	0.0525	0.0000

S.G.= Spotted Green, *=Significant.

Organization of the genetic diversity of *Hibiscus cannabinus*

The radial representation of the dendrogram of the entire collection of *H. cannabinus*, constructed from the dissimilarity matrix according to the Neighbor-Joining method gives a distribution of accessions, in three genetic groups A, B and C (Fig. 2). In genetic group A, constituted of 19 individuals, 4 morphotypes are distinguished, namely purple, spotted-green and mostly green and purple. These individuals come mainly from 2 provinces of the country including 3 of Kadiogo and 16 of Koupéla. Group B, highly composite contains the largest number of accessions, namely 34, divided into 3 subgroups including B_1, B_2, and B_3. Subgroup B_1 contains 16 accessions exclusively from Nahouri Province. There are 2 morphotypes namely green mostly and black. Subgroup B_2 consists of 15 accessions and subgroup B_3 has 3 accessions. Group B accessions consist of almost all the morphotypes except the spotted-green and come from 2 provinces (Nahouri and Sanguié). Finally, group C consists of 17 individuals. All accessions of Sissili are there.

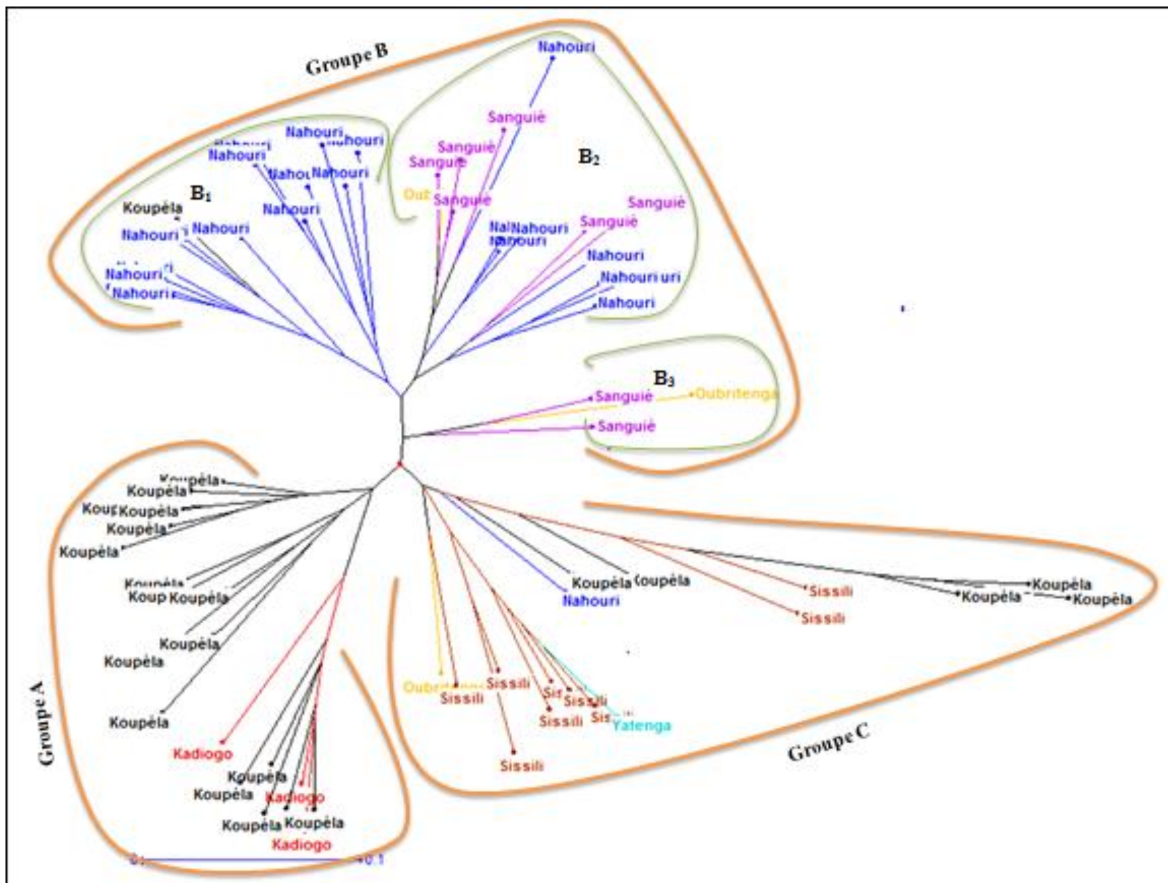

Fig. 2: Tree representation of the dendrogram based on the dissimilarity matrix obtained for all 70 accessions according to the provenance of the accessions with the "Neighbor-Joining" method.

Organization of the genetic diversity of *Hibiscus cannabinus*

Description of the genetic groups of *H. cannabinus*

The genetic parameters of the three genetic groups obtained from the Neighbor-Joining dissimilarity matrix method are shown in Table 5. Group A has the highest genetic parameters with an effective number of alleles of 4.46; 9 private alleles, an expected heterozygosity of 0.295, a Shannon diversity index (I) of 3.584, a polymorphic information content of 0.305 and 89.22% of polymorphic loci. Group B has the lowest number of effective alleles (4.04), the lowest expected

heterozygosity (0.220), the lowest Shannon diversity index (2.728), and the lowest polymorphism information content (0.229). However, it contains a number of private alleles and a polymorphic loci rate, higher than group C. Group C, on the other hand, does not have any private alleles but it contains genetic parameters with intermediate values to those of the groups A and B.

Table 5. Distribution of genetic diversity by genetic group.

Genetic group	A_e	A^P	H_e	I	PIC	P (%)
Group A	4.46	9	0.295	3.584	0.305	89.22
Group B	4.06	4	0.220	2.728	0.229	73.53
Group C	4.31	0	0.257	3.056	0.277	68.63
Average	4.28	4	0.257	3.122	0.270	77.12

Ae: effective number of alleles, Ap: number of private alleles, He: expected heterozygosity, I: Shannon diversity index, PIC: polymorphic information potential, P (%): Polymorphic loci rate.

Genetic distance between genetic groups

The genetic distances between the three genetic groups revealed by the minimal Nei distance and the intergroup genetic differentiation (Table 6) show that the three genetic groups differ from each other very significantly. Groups A and B are the most distant (distance = 0.13). The differentiation index Fst reveals instead that the group B and C are the most distinct (Fst = 0.324), followed by groups A and C (Fst = 0.256), A and B (Fst = 0.2317), which are also different significantly.

Table 6. Genetic differentiation between genetic groups.

Genetic group	Minimal Nei Distance			Differentiation index Fst		
	Group A	Group B	Group C	Group A	Group B	Group C
Group A	0.000			0.0000		
Group B	0.129	0.000		0.2317**	0.0000	
Group C	0.094	0.091	0.000	0.2560**	0.3240**	0.0000

**=Highly significant.

Influence of different factors on the structuring of *H. cannabinus* genetic diversity

The study of the effect of the factors "morphotype" and "genetic group" in Table 7 shows that each of them exerts, at different levels, an influence on the genetic differentiation of the *H. cannabinus* collection. Thus, the factor "genetic group", with a differentiation index Fst of 0.3240, alone accounts for 32.40% of the total variability. It influences more diversity. On the other hand, the "morphotype" factor with a differentiation index (Fst = 0.2258) accounts for only 22.58% of the total variability and influences this genetic diversity less than the genetic group factor.

Table 7. Effect of "morphotype" and "genetic group" factors on the genetic differentiation of the *H. cannabinus* collection.

Factors	Minimal Nei distance	Differentiation index (Fst)	Variability rate (%)
Morphotypes	0.276	0.2258**	22.58
Genetic groups	0.129	0.3240**	32.40

Discussion

The SSR markers used revealed genetic diversity among accessions from the Burkina Faso *Hibiscus cannabinus* collection. The high polymorphism rate of 96.08% obtained would be related to the discriminatory ability of SSR markers to reveal the genetic diversity of the *Hibiscus cannabinus* collection studied. Indeed, according to Ould Ahmed et al. (2010), a very high level of polymorphism testifies to the high level of polymorphism in accessions and the effectiveness of markers used to assess genetic diversity within the species. Furthermore, the polymorphism information content (PIC) that indicates not only the number of alleles detected but also the relative

frequency of these alleles is an important means of estimating genetic diversity (Smith et al., 2000). As a result, the average value of PIC (0.311) lower than that (PIC = 0.44) obtained by Karan et al. (2013) on hybrids of *H. cannabinus* and *H. radiatus* reflects a low genetic diversity of the collection compared to that of Karan et al. (2013). This low genetic diversity is thought to be related to the selection pressure on the size and origin of the accessions used in this study. Indeed, the studies of Karan et al., 2013 focused on 73 hybrids of various origins. According to (Nebié, 2014) genetic diversity is highly dependent on sample size, type and collection area, and the number and type of microsatellite markers used. The heterozygosity (He) level of 0.31 is higher than that obtained by Phumichai et al. (2014), of 0.095 and 0.143 respectively on *Corchorus capsularis* and *Corchorus olitorius* with the same markers. This may be due to a higher genetic diversity within accessions *of H. cannabinus* compared to *C. olitoruis* and *C. capsularis* which resulted in a high heterozygote richness. This genetic diversity is confirmed by the high mean values of the Shannon diversity index (I) and the effective number of alleles (Ae) of 3.73 and 3.87 respectively. This result is probably related to the partially allogamous reproduction regime of up to 70%, ie 30% autogamy (Vogel JM, Rafalski A, 1996). However, this partial allogamy offers opportunities for cross-fertilization which favors a natural genetic mix leading to a large genetic diversity (Demarly et al., 1996). Moreover, the structuring of genetic diversity into three groups without taking into account the climatic zone could be explained by the methods of seed management by producers, namely mixed farming, seed exchange or the introduction of other crop cultivars by the migratory flow (Kiébré et al., 2017). In fact, during their migration, the populations move with their seeds which they introduce in the localities of receptions. As a result, the same cultivars are found in several crop areas over distances of 100 to 800 km (Diouf et al., 2007). In addition, Group B is the most diverse. This could be due to the fact that this group is composed of accessions of diverse and therefore very diverse origin. On the other hand, group A, made up of only 16 Koupéla accessions and 3 Kadiogo accessions, is the most homogeneous, thus showing a weak genetic base between the accessions of this group. This could be justified by the exchange of seeds between producers. The weak differentiation between the morphotypes suggests that certain morphological and phenological characteristics do not necessarily derive from the expression of the gene but often from the effect of the environment. As a result, although phenotypic traits are essential for the assessment of diversity, they are often influenced by environmental factors, which do not allow a better understanding of diversity (Banerjee et al., 2012). Similarly, these results accurately justify the three morphotypes obtained during the ethnobotanical survey, which indicated that farmers did not distinguish red from purple and black from purple (Kabré Z, 2016). The same interpretation was obtained by Kiébré Z et al. (2015) on white Caya morphotypes (*Cleome gynandra* L.). So the criteria used in peasant denominations are often a reliable means of distinguishing different local varieties. These genetic groups are therefore genetically different. According to Ould Ahmed et al. (2010), total genetic diversity is the sum of intra and inter-population diversity. This inter-population genetic diversity is described by Wright's (1951) genetic differentiation index (Fst), which is the correlation between two randomly drawn alleles from two different subpopulations. Then these significant values of Fst obtained, translate a high genetic variability within the population of *H. cannabinus* studied. Similar observations have been reported by Kalia et al. (2011) and (Siepe et al., 1997) respectively on collections of kenaf (*H. cannabinus*) Indian and exotic. The influence of "morphotype" and "genetic group" factors on the level and organization of genetic diversity is very high. In fact, the "genetic group" factor, with an Fst differentiation index of 0.3240, alone accounts for 32.40% of the total variability, compared with 22.58% for the "morphotype" factor. These results show that much of the total genetic variability of the *H. cannabinus* population studied is more attributable to the genetic group factor.

Conclusion

The seventeen SSR markers used for the molecular characterization of *H. cannabinus* from Burkina Faso, revealed genetic diversity in the *H. cannabinus* collection with a polymorphism rate of 96.08%. Two factors influenced this genetic diversity, including the"morphotype" and "genetic group" factors. A structuring of diversity into three

genetic groups has been established. Markers MJM217, MJM317, MJM606, and MJM618 were the most informative on this diversity and the most discriminating. These results show that the molecular markers used are appropriate for the evaluation of the genetic diversity of *H. cannabinus* from Burkina Faso.

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Conflict of interest statement

Authors declare that they have no conflict of interest.

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