Spatial scale patterns of Genetic diversity and Gene flow in populations of sweet detar (*Detarium microcarpum* Guill. & Perr.; Fabaceae)

Abstract

The main objective of this study is to investigate the patterns of genetic diversity and phylogenetic relationships within populations of *Detarium microcarpum* (Fabaceae) relative to different spatial conditions. Seventy-eight (78) accessions of *D. microcarpum* belonging to six populations (Phytogeographic districts) were sampled. In order to have very good quality DNA for molecular analysis, an optimization of the DNA isolation protocol was made. The molecular analysis of the accessions was carried out using 7 chloroplast microsatellite markers. The polymorphism rate (P) is 85.71% and the Polymorphism Information Content (PIC) was in the range of 0.43 (Ntcp_9) to 0.73 (Ccmp_2) with an average of 0.59. Allelic richness (A) ranged from 1.41 to 2.85 with an average of 2.04. The observed heterozygosity (Ho) ranged from 0.23 to 0.60 with an average of 0.39. The expected heterozygosity (He) ranged from 0.43 to 0.60 with a mean of 0.50. Wright's fixation index (F_{IS}) ranged from -0.17 to 0.47. The effective allele (Ae) is between 1.77 and 2.53 with an average of 2.02. Wright differentiation index (F_{ST}) was 0.024. Phylogenetic analysis revealed that the N_{ST} value was significantly higher than the G_{ST} value (N_{ST} = 0.452; G_{ST} = 0.190; $p < 0.05$). A relatively low h_d haplotype diversity is obtained (H_d = 0.320). AMOVA analysis showed that 17.35% of the variation existed within populations but 45.80% among populations within the species. Neighbor-Joining phylogenetic tree of *D. microcarpum* revealed three non-distinct clusters haplotypes showing the existence of gene flow between populations of the species. Our findings of genetic structure and gene flow of *D. microcarpum* populations based on different spatial conditions is caused by evolutionary forces such as scattering and pollination.

Keywords: Benin; cpDNA; genetic differentiation; microsatellite markers; phylogenetic relationship, sweet dattock

INTRODUCTION

Detarium microcarpum (Fabaceae) is a diploid forest plant species (2n = 22 chromosomes) used in human and animal nutrition, traditional medicine, crafts, household energy and also for medico-magic purposes [1, 2]. It is involved in the treatment of several diseases including sexually transmitted diseases. The flavonoids in methanol extracts of the plant have been shown to have potent inhibitory effects on HIV-1 or HIV-2 infection [3, 4]. *Detarium microcarpum* (sweet dattock or sweet detar) therefore constitutes an important source of income for the local communities which exploit its resources daily for their needs.

Unfortunately, due to overexploitation of its timber and the effects of climate change, the species become vulnerable in parts of West Africa [5, 6]. It is therefore urgent to develop strategies for its conservation in order to avoid its disappearance. The conservation of a forest species requires knowing the information about the level of genetic diversity and the extent of genetic differentiation within and between natural populations of that species [7]. Several studies have been carried out on systematics and parataxonomy [8], ethnobotany [1, 2], spatial and temporal distribution [9], phenology and morphological variability [10, 11] and pharmacology [12] of *D. microcarpum*. However, studies relating to the assessment of its genetic diversity using molecular genetic markers remain unavailable. Furthermore, knowledge of the level of genetic diversity and of the population structure of a plant is one of the very important aspects in programs for the management, conservation or genetic improvement of genetic resources [13, 14].

The development of effective strategies for the conservation and sustainable management of forest genetic resources requires an analysis of the diversity and genetic structure of these plant species which requires the use of highly informative genetic molecular

markers [15]. DNA molecular markers, including RAPD [16, 17], AFLP [18, 7] and RFLP [19, 20], were used to assess genetic diversity at the molecular level of many populations of plant species. In recent years, microsatellite markers (Simple Sequence Repeat, SSR) have become popular and powerful tools used for the assessment of genetic diversity due to their codominance, high rate of polymorphism, and putative influence on transcribed genes [21, 22]. Several nuclear microsatellite markers (SSRs) have been used to assess the genetic diversity of many plant species in Benin, including *Sorghum bicolor* [13], *Pennisetum glaucum* [23]. In addition, SSRs markers developed on chloroplast genomes (SSRcp) constitute a powerful tool in the analysis of phylogenetic relationships because they provide information on the structure of variability and allow the flow of genes to be traced back to the scale of the natural area [24, 25]. They make it possible to assess the impact of Human and animal activities on the diversity of species at the level of populations and sub-populations studied [26, 27]. As of today, no molecular genetic markers have been developed on *D. microcarpum*. Genetic characterization and assessment of the genetic diversity of a species involves the extraction of the genomic DNA of that species. However, DNA extraction kits are very expensive and their use requires expertise. That's why it's necessary to establish a DNA extraction protocol. The effectiveness of DNA isolation protocols depends not only on the type of plant (woody or not) of the organ serving as biological material (leaves, root, flower, etc.) and on its state of conservation, but also of the chemical composition of the biological material; which means that several protocols have to be tested in order to retain the ideal [28, 29].

The main objective of this study is to investigate the patterns of genetic diversity and phylogenetic relationships within populations of *Detarium microcarpum* relative to different spatial conditions.

MATERIALS AND METHODS

Plant material sampling

The plant material consists of seventy-eight (78) samples of young leaves of *D. microcarpum* (taken from 78 trees) collected in six (06) phytogeographic districts in Benin (Bassila, Zou, South Borgou, North Borgou, Atacora Chain and Mekrou-Pendjari), at the rate of thirteen (13) trees per phytogeographic districts, spaced at least 50 meters apart. These phytogeographic districts belong to Sudanian and Guineo-Sudanian and were chosen on the basis of the occurrence of natural stands of the species (Fig. 1; Table 1) [9]. Samples are georeferenced using a Global Position System (GPS) and those belonging to the same phytogeographic district are considered to be from the same population (Table 2).

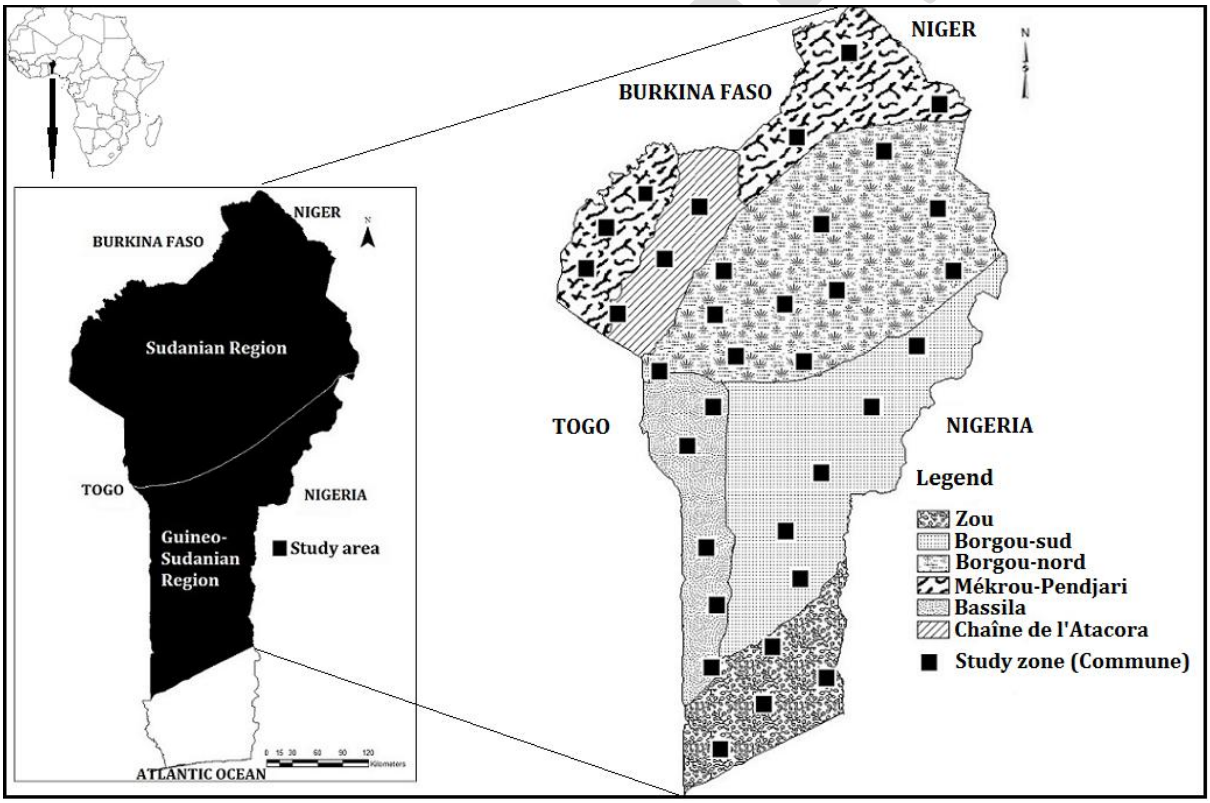

Fig. 1: Map of Benin republic showing the location of sampling areas.

Table 1: Ecological characterization of the climatic zone of sampling area.

Phytogeographic district	Climatic zone	Rainfall regime	Rainfall (mm)	Major soil types	Major plant formation

Bassila	Guineo-Sudanian	Tendency to unimodal	Min: 1100 Max: 1300	Ferrallitic soils with concretions and breastplates	Semi-deciduous forest, woodland, and riparian forest
Zou	Sudanian	Unimodal (1 rainy season)	Min: 1100 Max: 1200	Ferruginous soils on crystalline rocks	Dry forest, woodland, and riparian forest
South Borgou					
North Borgou					
Atacora Chain			Min: 1100 Max: 1200	Poorly evolved & mineral soils	Riparian forest, dry forest, and woodland
Mekrou- Pendjari			Min: 900 Max: 1000	Ferruginous soils with concretions on sedimentary rocks	Tree and Shrub savannahs, dry forest and riparian forest

Table 2: Study site and geographic coordinates for the sample of *D. microcarpum* populations.

Identifiers	Geographic coordinates		Population (Population id)	Identifiers	Geographic coordinates		Population (Population id)
	Lat (N)	Long (E)			Lat (N)	Long (E)	
BaBa174	9°24.197'	1°35.042'	Bassila (BA)	CaBo148	10°10.449'	1°11.949'	Atacora chain (AC)
BaBa176	9°21.467'	1°33.965'		CaBo154	10°10.311'	1°11.711'	
BaBa179	9°12.073'	1°33.985'		CaKe101	10°56.886'	2°12.880'	
BaBa180	8°57.805'	1°39.095'		CaKe102	10°56.175'	2°11.869'	
BaBn184	8°36.667'	1°40.691'		CaKe103	10°56.643'	2°12.135'	
BaBn188	8°16.007'	1°57.712'		CaKo155	10°18.278'	1°39.151'	
BaCo114	9°49.548'	1°33.283'		CaKo158	10°19.672'	1°42.664'	
BaCo116	9°52.274'	1°32.090'		CaNa119	10°02.685'	1°29.240'	
BaCo117	9°56.263'	1°31.824'		CaNa122	10°10.972'	1°24.796'	
BaDj164	9°41.364'	1°35.958'		CaNa124	10°21.805'	1°21.151'	
BaDj167	9°41.877'	1°30.041'		CaNa145	10°25.105'	1°21.715'	
BaDj169	9°40.455'	1°41.667'		CaTo126	10°28.387'	1°22.130'	
BaDj170	9°39.701'	1°42.187'		CaTo130	10°29.293'	1°22.543'	
BnBe63	9°50.575'	2°59.107'	North Borgou (NB)	MpBa100	11°15.101'	2°38.385'	Mekrou-Pendjari (MP)
BnBe64	10°00.937'	2°40.142'		MpBa97	11°18.339'	2°31.929'	
BnGo78	10°54.550'	2°51.288'		MpCo138	10°28.515'	1°02.096'	
BnKa111	10°40.175'	3°34.961'		MpCo139	10°30.047'	1°00.575'	
BnKa112	11°38.037'	3°34.894'		MpCo142	10°33.703'	0°59.623'	
BnKa113	10°28.458'	3°32.159'		MpMa143	11°44.326'	3°13.268	
BnKa81	11°05.820'	2°59.740'		MpMa144	11°41.418'	3°12.490'	
BnKa83	11°05.552'	3°00.054'		MpMa85	11°40.966'	3°12.099'	
BnPe161	10°14.913'	1°53.586'		MpMa88	11°39.939'	3°11.739'	

BnSe105	10°57.214'	3°25.377'		MpMa91	11°32.960'	3°07.250'	
BnSe108	10°50.773'	3°38.025'		MpMa94	11°30.346'	3°06.257'	
BnSi70	10°16.518'	2°27.544'		MpTa132	10°37.822'	1°17.967'	
BnSi73	10°21.275'	2°23.450'		MpTa135	10°39.378'	1°14.613'	
BsNd54	9°44.539'	2°41.790'	South Borgou (SB)	ZoDa28	7°48.352'	2°10.954'	Zou (ZO)
BsNd56	9°44.550'	2°41.753'		ZoDa29	7°59.986'	2°26.874'	
BsNi57	9°54.184'	2°55.997'		ZoDa30	7°57.564'	2°28.336'	
BsNi58	9°49.231'	2°59.327'		ZoDj1	7°30.848'	2°05.411'	
BsOu35	8°28.530'	2°26.392'		ZoDj2	7°31.037'	2°05.459'	
BsPa51	9°20.206'	2°36.978'		ZoDj3	7°31.511'	2°04.566'	
BsPa52	9°20.132'	2°34.349'		ZoDj6	7°31.546'	2°04.507'	
BsPe61	9°50.008'	2°59.302'		ZoDj13	7°29.755'	1°59.643'	
BsTc41	8°23.426'	2°29.306'		ZoDj15	7°29.771'	1°59.635'	
BsTc42	8°52.915'	2°34.969'		ZoDj23	7°29.520'	1°59.889'	
BsTc47	8°54.157'	2°35.799'		ZoGl31	8°01.233'	1°59.083'	
BsTc49	8°55.208'	2°35.397'		ZoSl187	7°55.353'	2°00.811'	
BsTc50	9°02.079'	2°36.925'		ZoSa34	8°28.551'	2°26.384'	

Optimization of genomic DNA isolation protocol of *D. microcarpum*

Molecular Genetics and Genome Analysis Laboratory of University of Abomey-Calavi (Benin) served as a study setting for molecular analysis of *D. microcarpum* samples. Several genomic DNA isolation protocols (Table 3) were tested on different quantities of young fresh leaves (0.1g, 0.15g and 0.2g) on the one hand and on dry young leaves on the other hand of *D. microcarpum*. The composition of the isolation buffer was adjusted in order to find the appropriate protocol, relatively fast, inexpensive and allowing an average yield of good quality genomic DNA.

Table 3: Composition of the buffer solutions of the different genomic DNA isolation protocols tested.

Protocol tested / **Composition**	**Dellaporta et al. [48]**	**Doyle & Doyle [49]**	**Agbangla et al. [51] (*Dioscorea spp.*)**	**Benbouza et al. [50] (*Gossypium spp.*)**	**Adjé et al. [29] (*Ananas cormosus*)**
MATAB (%)	-	-	4	-	-
CTAB (%)	-	2	-	2	2
Tris-HCl (mM), pH = 8	100	100	100	100	500
NaCl (M)	0.5	1.4	1.4	2	1.3
EDTA (mM)	50	20	20	20	5
Ammonium acetate (mM)	-	-	-	10	-

βmercapto ethanol (%)	0.07	0.5	-	5	0.1
Charcoal (mg.g^{-1})	-	-	-	10-15	-
SDS	20%	-	-	-	-
Incubation time at 65°C (min)	10	60	90	60	90

DNA quantification

The DNA yield was estimated using a spectrophotometer at UV-VIS 230, 260 and 280 nm. The purity of the DNA was determined by calculating the ratio A260/A280 nm to assess the protein contamination and A260/230 to assess polysaccharide contamination [30]. The DNA concentration was calculated with the following formula: [DNA] = A260 x DF x 50 µg/ml, where [DNA] is the DNA concentration, A260 is the absorbance at 260 nm, DF = Dilution Factor; 50 µg/ml is the concentration of DNA when A260 = 1.

DNA amplification by Polymerase Chain Reaction (PCR)

Dilutions of the DNA extracts were made to obtain the concentrations necessary for the amplification of DNA by PCR. A concentration of around 150 ng / µL was obtained from the suspensions previously obtained after DNA extraction. To amplify sequences of the *D. microcarpum* DNA, seven (07) chloroplast microsatellite markers (SSRcp) out of the ten tested were used. These are six (06) Ntcp primers (*Nicotiana tabacum* chloroplast) developed on tobacco [31] and a Ccmp (Consensus chloroplast microsatellite primers) primer, a universal primer developed by Weising and Gardner [32]. The primers used are: Ntcp_8, Ntcp_9, Ntcp_33, Ntcp_36, Ntcp_37, Ntcp_39 and Ccmp_2 (Table 4). These primers have been used successfully on other speculations such as *Helianthus annuus* [33], *Solenostemon rotundifolius* [26] and *Solanum tuberosum* [34]. Microsatellite primers from intergenic regions, introns and exons were used to assess the variability of the chloroplast DNA of *D. microcarpum* from the collection studied. Twenty two microliters (22 µL) of PCR master mix composed of 200 µM dNTP; 0.2 µM of each primer (forward and reverse); 1.25 µM of

MgCl2; 0.1U/µL of Taq DNA-polymerase; 5 µL of 5X Tris-HCl buffer and 9.25 µL of ultrapure water were added to 3 µL of DNA (approximately 3ng/ µL) of each sample. A Programmable thermal Blok II thermal cycler was used for amplification. The cycle of amplification included a pre-denaturation at 94°C for 4 min followed by 35 cycles, each cycle consisted of a denaturation at 94°C for 30 s, hybridization in the appropriate temperature (50°C or 60°C, Table 4) for 1 min and an elongation at 72°C for 1 min. A final incubation at 72°C for 8 min ended the program. The effectiveness of the amplification was tested by electrophoresis on 2% agarose gel in 0.5X TBE buffer. Gels were run in horizontal gel system at 100 V for 30 min and later photographed under UV light after be stained with ethidium bromide (BET).

Table 4: Characteristics of the chloroplastic microsatellite primers.

SSRs Loci	Gene location of microsatellite repeat(s)	Repeat unit	Forward primer sequence (F) Reverse primer sequence (R)	Ta (◦C)	Size in *N. tabacum* (pb)	PIC	Na
Ntcp_8	trnG intron	T11	F: ATATTGTTTTAGCTCGGTGG R: TCATTCGGCTCCTTTATG	55	251	0.52	3
Ntcp_9	trnG/trnR intergenic region	T10	F: CTTCCAAGCTAACGATGC R: CTGTCCTATCCATTAGACAATG	55	237	0.43	2
Ntcp_33	*rpo*A exon	T10	F: TGGCTGTTATTCAAAAGGTC R: CATGATAAATTGGCTAAACTCA	60	149	-	1
Ntcp_36	rps19/rpl2 intergenic region	T14	F: GTAGTAAATAGGAGAGAAAATAG R: ATGATACATAGTGCGATACAG	50	125	0.59	3
Ntcp_37	rrn5/trnR intergenic region	A13	F: TTCCGAGGTGTGAAGTGG R: CAGGATGATAAAAAGCTTAACAC	55	143	0.65	3
Ntcp_39	trnR/rrn5 intergenic region	T13	F: GTCACAATTGGGGTTTTGAATA R: GACGATACTGTAGGGGAGGTC	60	156	0.64	3
Ccmp_2	5′ to trnS intergenic region	A11	F: GATCCCGGACGTAATCCTG R: ATCGTACCGAGGGTTCGAAT	60	189	0.73	7

Ccmp: consensus chloroplast microsatellite primers; Ntcp: *Nicotiana tabacum* chloroplast. Ta: annealing temperature; PIC: Polymorphism Information Content; Na: number of different alleles.

Electrophoresis and revelation of PCR amplification products

The determination of the polymorphism at each locus was carried out by migration of the PCR amplification products by electrophoresis in 5% denaturing polyacrylamide gel of

305 × 385 mm (5% acrylamide-bisacrylamide (19:1), 8 mol urea in Tris-borate-EDTA/L (TBE) buffer, pH 8) at constant power of 60 W for 1 h 30 min to 2 h, depending on the expected product size. The detection of electrophoretic plates was carried out with silver nitrate ($AgNO_3$) according to Creste et al. [35]. The different bands or allelic level are determined by scoring as a function of the level of migration of the bands obtained.

Data analysis

The bands obtained at each locus were recorded as allelic compositions: absence and presence of band were respectively coded as 0 and 1. A binary matrix was constructed for statistical analysis.

To analyze the effectiveness of the microsatellite markers used in characterizing *D. microcarpum* populations, polymorphism rate (P) and Polymorphism Information Content (PIC) were calculated. In order to assess the genetic differentiation and the genetic structure within and between populations of *D. microcarpum*, following genetic diversity and structure parameters are calculated: Allelic richness (A), Effective allele (Ae), Expected heterozygosity (He), Observed heterozygosity (Ho), Wright's fixation index (F_{IS}) and Wright's differentiation index (F_{ST}).

Polymorphism rate (P) is the quotient of the number of polymorphic markers (Mp) over the total number of markers (Mt): P = Mp/Mt x 100. A population is polymorphic for a locus, if the allelic frequency of the most frequent allele is less than 0.95 [23].

Polymorphism Information Content of (PIC) was computed by the formula: $PIC = 1-\sum fi^2$ where *fi*, the allele frequency. It provides information on the discriminating power of the locus [13]. Allelic richness (A) is the sum of alleles for a locus over the total number of loci. It is determined by the formula: $A = (1/k)\sum_{n=1}^{k} ni$ where *k* is the total number of loci and *ni* the number of allele per locus [36]. Allelic richness is particularly important for conservation

strategies because it is often used in the management of collections and seed banks [37]. Effective allele number (Ae) was computed by the formula: $Ae = 1 / \Sigma(fi)^2$ where *fi* is the allele frequency. It provides information about the dispersal ability of the organism and the degree of isolation among populations [38]. Rate of expected heterozygosity (He) or genetic diversity of Nei was calculated by locus according to the formula: $He = 1-\sum (fi)^2$, where *fi* is the frequency of the allele *i* at the considered locus [39]. The average expected heterozygosity rate of the loci was calculated by population and also by chromosomal region, in order to determine the level of genetic variability of the microsatellite sequences of the coding and non-coding regions of the chloroplastic DNA (cpDNA) of *D. microcarpum* [40]. Rate of observed heterozygosity (Ho) was calculated from the measured frequency of heterozygotes by:

Ho = Nh/(Nt) where *Nh* is the number of heterozygous individuals and *Nt* the total number of individuals [39]. By comparing these two indices (He and Ho), it is possible to deduce the impact of evolutionary forces in populations, due to self-fertilization or autogamy. Wright fixation Index (F_{IS}) was calculated from the allele frequencies under the Hardy-Weinberg hypothesis according to the formula: $F_{IS} = 1 - (Ho / He)$. It reflects the differentiation of individuals within populations. $F_{IS} = 1$ mean complete fixation (self-fertilization), $F_{IS} <1$: excess heterozygosity, $F_{IS} = 0$: Hardy-Weinberg equilibrium population. $F_{IS} <0$: excess heterozygosity [38, 41]. Wright differentiation index (F_{ST}) is related to heterozygosity and genetic drift. Since inbreeding increases the frequency of homozygotes, as a consequence, it decreases the frequency of heterozygotes and genetic diversity. It is commonly used to determine whether there is gene flow between populations. It is obtained with the following formula: $F_{ST} = 1 - (H_S / H_T)$. Where Hs is the average He of subpopulations assuming random mating within each population, and H_T is the *H*e of the total population assuming random mating within subpopulations and no divergence of allele frequencies among subpopulations. According to Wright [41], $0 < F_{ST} < 0.05$: weak differentiation; $0.05 < F_{ST} < 0.15$: moderate

differentiation; 0.15 < F_{ST} < 0.25: significant differentiation; F_{ST} > 0.25: very important differentiation. In order to establish the phylogenetic relationship within populations of *D. microcarpum* with a quantification of gene flow in its populations, the program PermutCpSSR_1.2.1 [42] was used to calculate the level of population differentiation at the species level (G_{ST}), and an estimate of population subdivisions for phylogenetically ordered alleles (N_{ST}). G_{ST} and N_{ST} are often used to assess the geographical structure affecting population differentiation [43]. If there is a phylogenetically matched haplotype, the closest haplotypes are more often found mixed in the same populations. Differentiation measured by taking into account similarities between haplotypes (N_{ST}) is superior to differentiation based only on haplotype frequency (G_{ST}). The analyses of molecular variance (AMOVA) in ARLEQUIN 3.5 (available at http://cmpg.unibe.ch/software/arlequin3/) were performed to calculate the genetic variation among populations and within populations, using a significance test based on 1,000 permutations [44]. A classification of accessions was made from the genetic distance calculated by the dissimilarity coefficient of Jaccard with 1000 bootstraps repetitions and a dendrogram was built by the Neighbor-Joining method with DARwin version 6 software [45].

RESULTS

Genomic DNA isolation buffer of the new protocol

Table 3 showed the composition of the buffer solutions of the different genomic DNA isolation protocols tested. Genomic DNA isolation buffer of the new protocol is composed of: Chloroform Isoamylic Alcohol (24: 1), isopropanol, 70% ethanol, Tris-EDTA (10 mM Tris-HCl, 1 mM EDTA) and 2% CTAB (Cetyl Triammonium Bromide) containing 2g of CTAB, 1.4M of NaCl, 100mM of Tris-HCl (1M, pH = 8), 20mM of EDTA (0.5M, pH = 8) supplemented with 2% β-Mercaptoethanol and 0.5% Proteinase K (added after sterilization and just before use in a hood).

New protocol for genomic DNA isolation from *D. microcarpum*

The new protocol resulted in cleaner, good quality DNA that was easily amplified by PCR (Fig. 3). This new protocol is described as follows: A quantity of 150 mg of dry leaves per sample was weighed and ground in a porcelain mortar with 1 mL of CTAB buffer supplemented with 2% β-Mercaptoethanol and 0.5% proteinase K. The ground material obtained was poured into a 2 mL eppendorf tube, homogenized for a few minutes and then incubated in a water bath at 65 °C with gentle stirring for 60 min with homogenizations every 10 min. 1 mL of Chloroform Isoamylic Alcohol (CIA) buffer was added to the mixture after cooling followed by gentle stirring for 5 min. The whole is centrifuged at 10,000 rpm for 15 min at 4 °C without brake (free deceleration). The upper phase aqueous supernatant is transferred to another 1.5 mL eppendorf tube to which an equal volume (approximately 1 mL) of very cold isopropanol has been added to precipitate the DNA. In order to optimize the quantity of the DNA ball, an incubation is done at -20 °C for 30 min. After this precipitation phase, the mixture was centrifuged at 10,000 rpm for 10 min. The supernatant was removed and on the pellet, 500 µL of 70% ethanol was added and the mixture homogenized then left at room temperature for 20 min. The mixture was then centrifuged at 10,000 rpm for 10 min then the supernatant carefully emptied and the DNA pellet dried at room temperature on filter paper in order to remove the ethanol (Fig. 2). After drying, the DNA ball was taken up in 50 µL of Tris-EDTA buffer. To be sure of the success of the DNA extraction, the DNA yield was estimated using a spectrophotometer at UV-VIS 230, 260 and 280 nm, and 2 µL of genomic DNA extract were visualized on a 1% agarose gel stained with ethidium bromide (BET). After this confirmation, DNA samples were kept in a freezer at -20°C for molecular analysis. The shape and the relative intensity of the agarose gel migration bands are well parallel to the DNA concentration values extracted for the different samples.

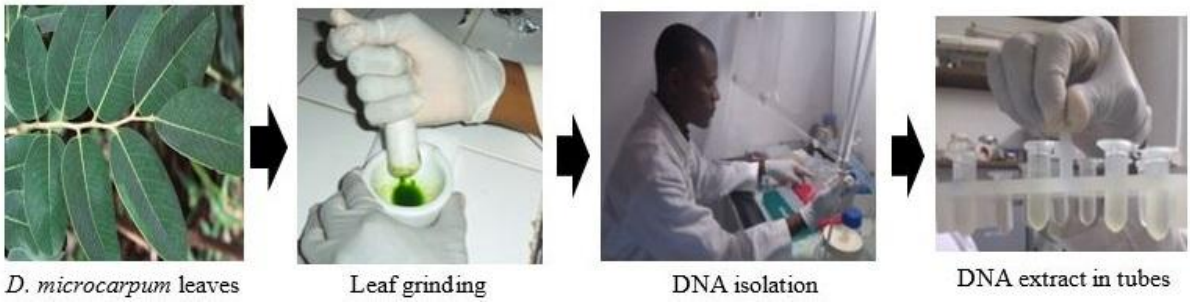

Fig. 2: Some steps in the classic process of isolation *Detarium microcarpum* genomic DNA.

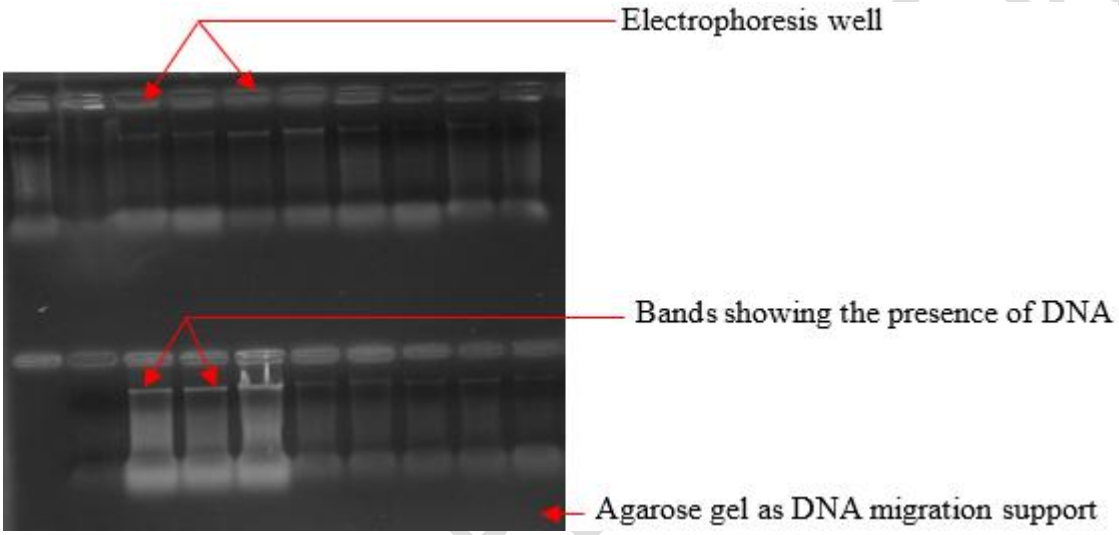

Fig. 3: Analytical electrophoresis profiles on agarose gel of genomic DNA from some *Detarium microcarpum* samples.

Genetic structure of *D. microcarpum* using SSR markers

Seven chloroplast microsatellite markers (Ntcp_8, Ntcp_9, Ntcp_33, Ntcp_36, Ntcp_37, Ntcp_39 and Ccmp_2) of ten tested for DNA amplification by PCR amplified *D. microcarpum* genome and were polymorphic except Ntcp_33 (which was monomorphic). Polymorphism rate (P) was so 85.71%. Four out of ten (Ntcp_5, Ntcp_15, Ntcp_25 and Ntcp_26) of the primers developed on *N. tabacum* failed to amplify a specific region on cpDNA in *D. microcarpum* accessions. On the chloroplast genome of *N. tabacum* these primers amplify microsatellite loci at T14 (Ntcp_5), at A10 (Ntcp_15), at A13 (Ntcp_25) and

at T10 (Ntcp_26) with bands whose size varies between 120 and 190 bp. In total, twenty-two (22) alleles were detected on the seven amplified chloroplast microsatellite loci on *D. microcarpum* genome. The number of alleles per locus (Na) varied from 1 (Ntcp_33) to 7 (Ccmp_2) with an average of 3.14. Major allele frequency varied from 0.12 (Ntcp_9) to 0.22 (Ntcp_37) with an average of 0.16 (Fig. 4). Ccmp_2 locus was the most polymorphic (Na = 7 alleles) and the most discriminating (PIC = 0.73) while Ntcp_9 was the least polymorphic (Na = 2 alleles) and the least discriminating (0.43) (Table 4). The Polymorphism Information Content (PIC) ranged from 0.43 to 0.73 with an average of 0.59. Within the phytogeographic districts, the allelic richness (A) varied from 1.41 (Bassila) to 2.86 (South Borgou) with an average of 2.04. The observed heterozygosity (Ho) ranged from 0.23 (Bassila) to 0.60 (Atacora chain) with an average of 0.39. The expected heterozygosity (He) ranged from 0.43 (Bassila) to 0.60 (South Borgou) with an average of 0.50. Wright's fixation index (F_{IS}) ranged from -0.17 (Atacora chain) to 0.47 (Bassila). Effective allele (Ae) is between 1.77 (Bassila) and 2.53 (South Borgou) with an average of 2.02 (Table 5).

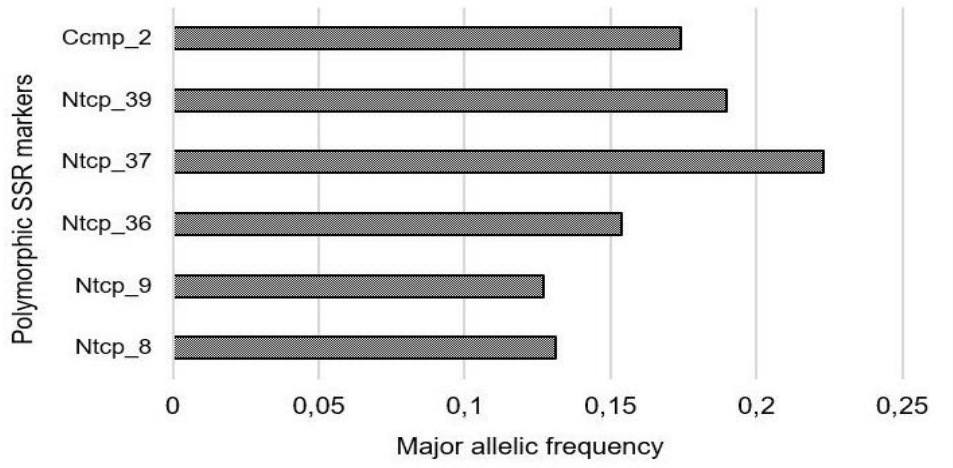

Fig. 4: Major allelic frequency of polymorphic SSR marker.

Table 5: Genetic diversity of *D. microcarpum* populations in Benin.

Population	N	A	Ae	Heterosigosity		F_{IS}
				Ho	He	
AC	13	2.000	2.053	0.603	0.513	-0.175
BA	13	1.428	1.769	0.230	0.435	0.471
MP	13	1.857	1.776	0.297	0.437	0.321
NB	13	2.428	2.092	0.425	0.522	0.186
SB	13	2.857	2.531	0.455	0.605	0.248
ZO	13	1.714	1.923	0.324	0.480	0.325
Mean	**13**	**2.047**	**2.024**	**0.389**	**0.498**	**0.229**

N: sampling collection size per population, A: allelic richness, Ae: effective allele, Ho: observed heterozygosity, He: expected heterozygosity, F_{IS}: Wright's fixation index.

Phylogenetic relationship and the level of gene flow in the populations of *D. microcarpum*

Genetic variability of the microsatellite sequences in the coding and non-coding regions was estimated on the basis of their expected heterozygosity. Wright differentiation Index (F_{ST}) is 0.024. Phylogenetic analysis revealed that the N_{ST} value was significantly higher than the G_{ST} value (N_{ST} = 0.452; G_{ST} = 0.190; P <0.05). A relatively low h_d haplotype diversity is obtained (H_d = 0.320). AMOVA analysis showed that 17.35% of the variation existed within populations but 45.80% among populations within the species (Table 6). The classification of *D. microcarpum* accessions showed on the phylogenetic tree constructed by the Neighbor-Joining method three (03) non-distinct Haplotypes characterized by the presence of accessions from different populations (Fig. 5). The distribution of individuals was therefore not made according to the populations considered. There is a mixture of individuals from populations in each haplotype.

Table 6: AMOVA result for *D. microcarpum* populations from cpDNA haplotypes.

Source of Variation	Degree of Freedom	Sum of Squares	Variance Components	Percentage of variation	p Value
Among populations	5	50.175	3.246	45.80%	< 0.01
Within populations	14	48.307	0.584	17.35%	< 0.01

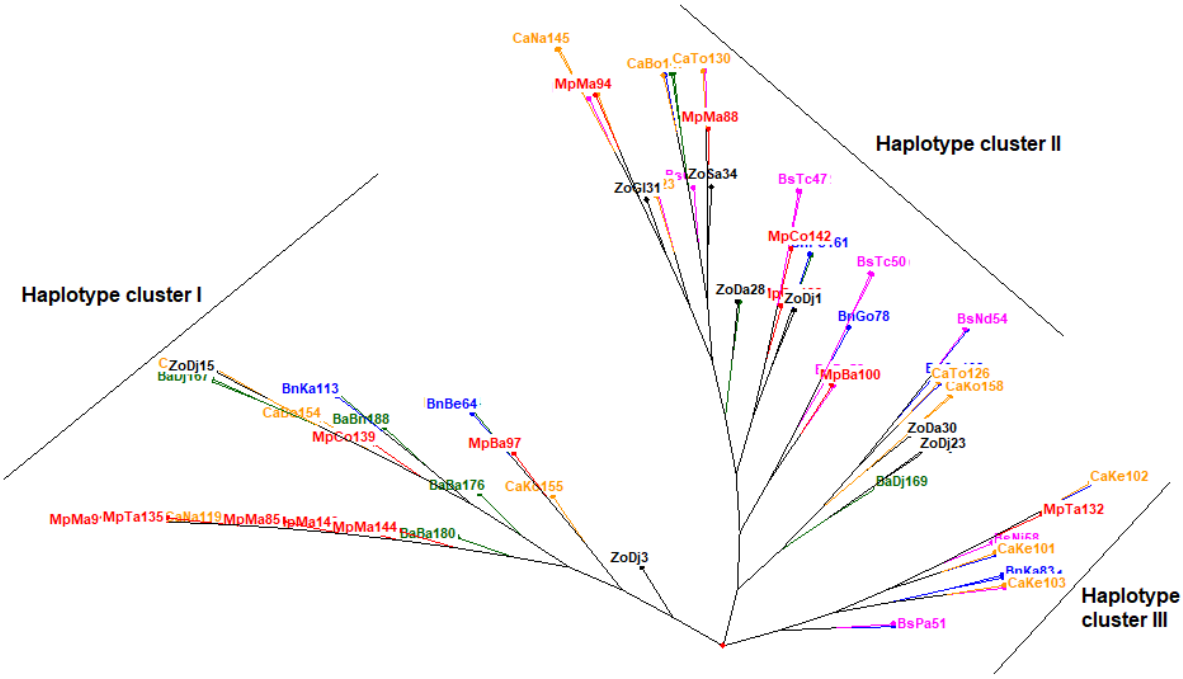

Fig. 5: Neighbor-joining phylogenetic tree showing haplotypes clusters from *Detarium microcarpum* populations.

Codes in the same colour are trees of *D. microcarpum* belonging to the same population.

DISCUSSION

Genomic DNA isolation of *D. microcarpum*: an essential step in genetic molecular analysis

DNA isolation is a crucial and the first step of a DNA-based genetic characterization study. The quality and purity of nucleic acids are among the most critical factors for subsequent analysis. Like some plants including *Ceratonia siliqua* [46], *D. microcarpum* is a leguminoseae very rich in secondary metabolites such as polysaccharides, polyphenols, tannins, proteins, etc. These secondary metabolisms can bind to the DNA molecule, making it difficult to extract and access enzymes [47]. That's why our first intentions and efforts in this study were concentrated on the development of an isolation technique capable of having a good quality of DNA which can be used as a template for the amplification of DNA by PCR. The deficiency of DNA isolation protocol adapted to *Detarium microcarpum* and the expensive cost of DNA extraction kits obliged us to test and modify some DNA isolation

protocols including that of Dellaporta et al. [48], Doyle and Doyle [49], Benbouza et al. [50], Adjé et al. [29] using Cetyl Triammonium Bromide (CTAB) and that of Agbangla et al. [51] using Mixed Alkyl Timethyl Ammonium Bromide (MATAB). The DNA isolation protocol set up by Dellaporta et al. [48] and Doyle and Doyle [49] using CTAB are very efficient and commonly used to extract genomic DNA from woody plants [46, 52]. However, their application to the leaf of *D. microcarpum* was inconclusive and the resulting bundle was either very viscous and insoluble or free from any trace of DNA. This led us to the various modifications to the protocol including the use of β-Mercaptoethanol and proteinase K considered as chemical agents having a positive and effective impact on the dissolution of the tannin-protein complex, responsible for the anchoring of several constituents cells of certain plants and the elimination of plant proteins [46]. The results of the DNA sample verification testify to the efficiency and cost-effectiveness of the modified Doyle and Doyle [49] protocol on *D. microcarpum*.

Amplification of cpDNA sequences by PCR

Chloroplast microsatellite primers tested on the accessions of *D. microcarpum* were developed on plant species belonging to the Solanaceae family with the aim of allowing their application in the study of the variability of chloroplast DNA in other species or families of Angiosperms [31, 32]. Region-specific amplification of *D. microcarpum* accessions cpDNA by these primers showed that genetic molecular markers developed in one species can be used in molecular studies of other species. However, the absence of amplification by some primers testifies to the limit of the universality of these primers as referred to, but above all the need to develop specific primers for each species of plant. This lack of amplification observed in the present study with certain primers was also observed in other species such as *Vitis vinifera* (Vitaceae), *Helianthus annuus* (Asteraceae), *Dioscorea sp*. (Dioscoreaceae) and *Solenostemon rotundifolius* (Lamiaceae) respectively by Imazio et al. [53], Wills & Burke [54], Chaïr et al.

[55] and Nanema et al. [26]. According to Decroocq et al. [56] and Nanema et al. [26], the lack of amplification of cpDNA sequences is explained by the absence of the corresponding microsatellite region targeted by the primer on chloroplast DNA.

Spatial genetic structuring and phylogeny between *D. microcarpum* populations

Molecular studies based on chloroplast DNA (cpDNA) focus on the one hand, the study of the mechanisms that control its evolution and on the other hand, the use of its variation as a tool to study the genetic diversity of plant species [57]. In Angiosperms, cpDNA is the most used in the study of phylogenetic relationships. Indeed, the small size of cpDNA, its abundance in the plant cell, its maternal single-parent inheritance and especially its conservative rate of evolution (no genetic mixing), make the chloroplast genome a privileged tool for phylogenetic studies [58, 59]. These properties of cpDNA and the characteristics of microsatellite markers make it possible to follow chloroplast gene flow and analyse the dynamics of genetic diversity of *D. microcarpum* at large time scales [60].

The highest values of the effective allele, the expected heterozygosity and allelic richness observed in the South Borgou phytogeographic district means that *D. microcarpum* has a dispersal capacity and a high degree of isolation in this population [38]. The relatively high value of the expected heterozygosity implies a high degree of dissimilarity within the species studied. Wright fixation index recorded in all the different populations are low ($F_{IS} < 1$). In addition, the F_{IS} calculated in the Atacora Chain phytogeographic district is negative ($F_{IS} < 0$) and reflects excess heterozygosity. These low rates of Wright fixation index can be explained by the high rate of allofertilization and / or dissemination that exists between the different individuals of *D. microcarpum* in the same population even though the species is autogamous [61]. Indeed, the seeds of *D. microcarpum* are mainly disseminated by humans, monkeys (*Cercopithecus spp*.), Rabbits (*Oryctolagus cuniculus*), squirrels (*Sciurus vulgaris*), giant rats (*Rattus spp*.), Elephants (*Loxodonta africana*) [2, 62]. This confirms the value of the

Wright differentiation index (F_{ST}) which is 0.024 ($F_{ST} < 0.05$). Results of AMOVA indicated that 17.35% of the total genetic diversity was due to differences within populations, while 45.80% of the overall genetic diversity was a result of differences among the population. These relatively low values of variation may be justified by the reproductive system of the species [63, 64] dispersion of the species among farmers [65], and evolutionary changes within the species. Haplotype diversity reflects the degree of difference among the haplotypes in each population. The low haplotype diversity is observed in the present study showed weak differentiation in populations of *D. microcarpum*. The permutation test revealed a higher value for N_{ST} (0.462) than G_{ST} (0.201), with $p < 0.05$, indicating a clear phylogenetic structure among the populations [66].

The presence of individuals belonging to different populations of *D. microcarpum* within the three haplotypes clusters could be justified by a probable common origin of these populations or the existence of gene flows between the different natural populations of the species. This finding could be explained by the flow of genes within individuals from one population to another. These results corroborate those of Fontaine et al. [67] who have shown that gene flow leads to the exchange of genes (or their alleles) between different populations. This gene flow observed in *D. microcarpum* populations is due to the mode of dispersal of seeds (dissemination) and pollens (pollination) of the plant by various agents. According to Kouyaté and Van Damme [10], the seeds of *D. microcarpum* are dispersed by wind (anemochory), animals such as mammals and birds (zoochory) and humans (anthropochory). Although the species is self-pollinating, it possesses a fraction of allogamy. Its flowers are pollinated by wind (anemogamy) and insects such as ladybugs, flies and bees (entomogamy). These various disseminators and pollinators contribute to a wide dispersion of seeds and pollens. This promotes a significant flow of genes within populations of the species. Indeed, the mode of seed dissemination and that of flower pollination are the main factors responsible for gene flow within a plant species.

CONCLUSION

The present study revealed distinct patterns of genetic diversity at different spatial scales. The new genomic DNA isolation protocol from *Detarium microcarpum* obtained after optimization is efficient and can be used successfully on other woody plants. Analysis of cpDNA using microsatellite markers showed a relatively high level of genetic polymorphism in accessions of the species within phytogeographic districts. There is an existence of gene flow within *D. microcarpum* populations in phytogeographic districts. Findings on the genetic structure and gene flow of *D. microcarpum* populations based on different spatial conditions are caused by evolutionary forces such as dispersal and pollination. This information is necessary for taking conservation measures for the species.

Availability of data and materials

The data sets supporting the conclusions of this article are included within the article.

Data Archiving Statement

This research contains no data that requires submission to a public database. Population names of *Detarium microcarpum* collection come from samples collected and georeferenced by the corresponding author in phytogeographic districts (Table 2). The names and allele sizes of the 7 SSR loci used in this study are listed in Table 4. Primer sequences for the SSR markers can be found in the original publications, which are referenced within the materials and methods of the manuscript.

COMPETING INTERESTS :

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

Disclaimer

This paper is an extended version of a preprint document of the same author.

The preprint document is available in this link: https://assets.researchsquare.com/files/rs-879012/v1/a4fd2ec1-a485-4071-a9be-f187e8eeea49.pdf?c=1637650977

[As per journal policy, pre-print article can be published as a journal article, provided it is not published in any other journal]

REFERENCES

1. Kouyaté AM, Van Damme P, De Meulenaer B, Diawara H. Contribution des produits de cueillette dans l'alimentation humaine. Cas de *Detarium microcarpum*. Afrika Focus. 2009;1(22):77-88.

2. Agbo IR, Vihotogbé R, Missihoun AA, Dagba AR, Assogbadjo EA, Agbangla C Indigenous knowledge of *Detarium microcarpum* Guill. & Perr. (Caesalpiniaceae) and implication for conservation in Benin (West Africa). Environment, Development and Sustainability. 2019a;22:626-6285. doi:10.1007/s10668-019-00477-3.

3. Mahmood N, Pizza C, Aquino R. De Tommasi N, Piacente S, Colman S, Burke A, Hay JA. Inhibition of VIH infection by flavonoïds. Antiviral Research. 1993;22:189–199. doi: 10.1016/0166-3542(93)90095-Z.

4. Nikiema JB, Simpore J, Sia D, Djierro K, Guissou IP, Kasilo OMJ. Introduction de plantes médicinales dans le traitement de l'infection à VIH : Une approche réussie au Burkina-Faso. The African Health Monitor. 2010;14.

5. Adjahossou SGC, Gouwakinnou GN, Houéhanou DT, Sode AI, Yaoitcha AS, Houinato MRB, Sinsin B. Efficacité des aires protégées dans la conservation d'habitats favorables prioritaires de ligneux de valeur au Bénin. Bois et forêts des tropiques. 2016;328(67-76):2.

6. Agbo IR, Missihoun AA, Vihotogbé R, Assogbadjo EA, Ahanhanzo C, Agbangla C. Impacts des usages traditionnels sur la vulnérabilité de *Detarium microcarpum* Guill. & Perr. (Caesalpiniaceae) dans le district phytogéographique Zou au Bénin. International Journal of Biological and Chemical Sciences. 2017;11(2):730-743.

7. Assogbadjo AE, Glèlè Kakaï R, Kyndt T, Sinsin B. Conservation genetics of baobab (*Adansonia digitata* L.) in the Parklands Agroforestry Systems of Benin (West Africa). Notulae Botanicae Horti Agrobotanici Cluj-Napoca. 2021;38(2):136-140.

8. Houénon GHA, Djossou AJ, Kouhinkpo EY, Salako KV, Tchobo PF, Adomou CA & Yédomonhan H. Parataxonomy, perceived dynamics and diversity of uses of two *Detarium* species in Benin (West Africa). Genetic Resources and Crop Evolution. 2021;2627–2654. https://doi.org/10.1007/s10722-021-01169-6.

9. Agbo IR, Idohou R, Vihotogbé R, Missihoun AA, Dagba RA, Assogbadjo EA, Agbangla C. Spatio-temporal dynamics of suitable habitats for *Detarium microcarpum* Guill. & Perr. (Caesalpiniaceae), a priority food tree species in Benin (West Africa). Model Earth Syst Environ. Environment, Development and Sustainability. 2019b;5:595–604. doi:10.1007/s40808-018-0550-x.s.

10. Kouyaté AM & Van Damme P. Caractères morphologiques de *Detarium microcarpum* Guill. et Perr. au sud du Mali. Fruits. 2002;57:231-238.

11. Agbo IR, Missihoun AA, Dagba AR, Assogbadjo EA, Agbangla C. Morphological variability of *Detarium microcarpum* Guill. & Perr. (Caesalpiniaceae) in Benin, West Africa. Asian Journal of Research in Agriculture and Forestry. 2018;1(2)1-12 doi:10.9734/AJRAF/2018/41234.

12. Sani A, Agunu A, Danmalam UH, Hajara I. Pharmacognostic Studies of the Stem Bark of *Detarium microcarpum* – Guill. and Perr. (Fabaceae). Natural Products Chemistry & Research. 2014; S1:004 doi:10.4172/2329-6836.S1-004.

13. Missihoun AA, Adoukonou-Sagbadja H, Sedah P, Dagba AR, Ahanhanzo C, Agbangla C. Genetic diversity of *Sorghum bicolor* (L.) Moench landraces from Northwestern Benin as revealed by microsatellite markers. African Journal of Biotechnology. 2015;14(16):1342-1353.

14. Jahan T, Ali MA, Raihan MS, Rahman MM, Abdullah HM, Huda MN, Hasan M. Indigenous Mota-named coarse rice germplasm is distinct from fine-grained rice collected from south-central coastal Bangladesh as compared with the morphological descriptors and molecular markers. Brazilian Journal of Botany. 2020;43: 933-945. https://doi.org/10.1007/s40415-020-00646-z.

15. Liu J, Sun ZX, Chen YT and Jiang JM. Isolation and characterization of microsatellite loci from an endangered tree species, Toona ciliata var. pubescens. Genetic Molecular Reseach. 2021; 11:4411-4417. doi:10.4238/2012.

16. Ercisli S, Orhan E, Esitken A, Yildirim N, Agar G. Relationships among some cornelian cherry genotypes (*Cornus mas* L.) based on RAPD analysis. Genetic. Resources and Crop Evolution. 2008;55 (4)613-618.

17. Vanijajiva O. Genetic variability among durian (Durio zibethinus Murr.) cultivars in the Nonthaburi province, Thailand detected by RAPD analysis. Journal of Agricultural Technology. 2011;7:1105-1114.

18. Schenk FM, Thienpont C-N, Koopman MJW, Gilissen JWJL, Smulders MJM. Phylogenetic relationships in Betula (Betulaceae) based on AFLP markers. Tree Genetic and Genomes. 2008;4:911–924 doi: 10.1007/s11295-008-0162-0.

19. Bhattacharjee R, Bramel PJ, Hash CT, Kolesnikova-Allen MA, Khairwal IS. Assessment of genetic diversity within and between pearl millet landraces. Theoretical and Applied Genetics. 2002;105:666-673.

20. Yan Z, Denneboom C, Hattendorf A, Dolstra O, Debener T, Stam P, Visser PB. Construction of an integrated map of rose with AFLP, SSR, PK, RGA, RFLP, SCAR and morphological markers. Theoretical and Applied Genetics. 2005;110:766–777 https://doi.org/10.1007/s00122-004-1903-6.

21. Benemann DP, Machado LN, Argel LWP, Bianchi VJ, Oliveira AC, Maia LC, Peters JA. Identification, characterization and validation of SSR markers from the gerbera EST database. Plant Omics Journal. 2012;5:156-159.

22. Huda MN, Hasan M, Abdullah MH, Sarker U. Spatial distribution and genetic diversity of wild date palm (*Phoenix sylvestris*) growing in coastal Bangladesh. Tree Genet Genomes. 2019;15:3. doi:10.1007/s11295-018-1310-9.

23. Dagba AR, Missihoun AA, Sedah P, Agbo IR, Agbangla C. Genetic Diversity of Cultivated Millet Varieties (*Pennisetum glaucum* (l.) R. Br.) in Benin Using Microsatellite Markers (SSRs) and Impact of Farmer's Practices on their Genetic Structuring. Journal of Natural Product and Plant Resources. 2018;8:23-40.

24. de Cesare M, Hodkinson TR, Barth S. Chloroplast DNA markers (cpSSRs, SNPs) for *Miscanthus*, *Saccharum* and related grasses (Panicoideae, Poaceae). Molecular Breeding. 2010; 26:539–544 (2010). https://doi.org/10.1007/s11032-010-9451-z.

25. Phumichai C, Phumichai T, Wongkaew A. Novel Chloroplast Microsatellite (cpSSR) Markers for Genetic Diversity Assessment of Cultivated and Wild *Hevea* Rubber.

Journal Plant Molecular Biology Reporter. 2015;33:1486–1498. https://doi.org/10.1007/s11105-014-0850-x.

26. Nanema KR, Missihoun AA, Agbangla C, Ahanhanzo C, Traore ER, Bationo/Kando P, Sawadogo M, Zongo J-D. Etude de la relation phylogénétique entre trois morphotypes de *Solenostemon rotundifolius* (Poir J. K. Morton) originaires du Burkina Faso par les marqueurs microsatellites chloroplastiques (SSRcp). International Journal of Biological and Chemical Sciences. 2010;4:1922-1931.

27. Vihotogbé R, van den Berg RG, Missihoun AA, Sinsin B, Sosef MS. Genetic diversity of bitter and sweet African bush mango trees (*Irvingia* spp., Irvingiaceae) in West and Central Africa. African Journal of. Biotechnology. 2015;14:3062-3074. doi:10.5897/AJB12.2564.

28. Chabi Sika K, Kefela T, Adoukonou-Sagbadja H, Ahoton L, Saidou A, Baba-Moussa L, Baptiste JL, Kotconi OS, Gachomo WE. A simple and efficient genomic DNA extraction protocol for large scale genetic analyses of plant biological systems. Plant Gene. 2015;(1)43-45.

29. Adjé O, Achigan-Dako E, Agbangla C. Optimizing genomic DNA isolation in pineapple (*Ananas comosus* l.). Journal of Plant Breeding and Genetics. 2016; (4)11-18.

30. Wilson K and Walker J. Principles and Techniques of Biochemistry and Molecular Biology. Cambridge University Press. 2010.

31. Bryan GJ, McNicoll J, Ramsay G, Meyer RC, Jong WSD. Polymorphic simple sequence repeat markers in chloroplast genomes of Solanaceous plants. Theoretical and Applied Genetics. 1999; 99:859-867.

32. Weising K & Gardner RC. A set of conserved PCR primers for the analysis of simple sequence repeat polymorphism in chloroplast genome of dicotyledons angiosperms. Genome. 1999;42:9-19.

33. Wills DM, Hester ML, Aizhong L, Burke MJ. Chloroplast SSR polymorphisms in the Compositae and the mode of organellar inheritance in *Helianthus annuus*. Theoretical and Applied Genetics. 2005;110:941-947. doi:10.1007/s00122-004-1914-3.

34. Zeka D, Sedlak P, Sedlakova V, Vasek J, Domkarova J, Vejl P. CpDNA SSR polymorphism in secondary germplasm of potato maintained in Czech gene bank. Pakistan Journal of Agricultural Sciences. 2014;51:743-748.

35. Creste S, Neto AT, Figueira A. Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. Plant Molecular Biology Reporter. 2001;19:299-306.

36. Bhandari HR, Bhanu AN, Srivastava K, Singh MN, Shreya A, Hemantaranjan A. Assessment of genetic diversity in crop plants - an overview. Advances in Plants & Agriculture Research.2017. 7:279-286. doi: 10.15406/apar.2017.07.00255.

37. Bataillon TM, David JL, Schoen DJ. Neutral genetic markers and conservation genetics: Simulated germplasm collections. Genetics. 1996;114:409-417.

38. Govindaraj M, Vetriventhan M, Srinivasan M. Importance of Genetic Diversity Assessment in Crop Plants and Its Recent Advances: An Overview of Its Analytical Perspectives. Genetics Research International. 2015 ; 431-487. doi:10.1155/2015/431487.

39. Nei M. Molecular evolutionary genetics. Columbia University Press, New York. USA, 1987; 52p.

40. Zeka D, Sedlak P, Sedlakova V, Sharma K, Vejl P. Phenotype and molecular diversity evaluation of some wild 2n Solanum species (Super series Rotata). Chilean Journal of Agricultural Research. 2015;75:147-151.

41. Wright S (1978) Evolution and the genetics of populations. Vol. 4. Variability within and among Natural Populations. University of Chicago Press, USA. 1987.

42. Pons O, Petit RJ. Measwring and testing genetic differentiation with ordered versus unordered alleles. Genetics. 1996;144:1237-1245.

43. Sun Y, Yang H, Zhang O, Qin L, Li P, Lee J, Chen S, Rahman K, Kang T, Jia M. Genetic diversity and its conservation implications of *Vitex rotundifolia* (Lamiaceae) populations in East Asia. PeerJ. 2019. DOI 10.7717/peerj.6194.

44. Excoffier L, Lischer HE (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour 10:564 567 DOI 10.1111/j.1755-0998.2010.02847.x.

45. Perrier X, and Jacquemoud-Collet JP. DARwin6.0. 2010. http://darwin.cirad.fr/darwin.

46. Konaté I. Diversité Phénotypique et Moléculaire du Caroubier (*Ceratonia siliqua* L.) et des Bactéries Endophytes qui lui sont Associées. Thèse. Université Mohammed V-Agdal, Maroc. 2007 ; 196p.

47. Lakshmi M, Parani M, Parida A. Molecular phylogeny of mangroves IX: Molecular marker assisted intra-specific variation and species relationships in the Indian mangrove tribe Rhizophoreae. Aquatic Botany. 2002;74: 201–217.

48. Dellaporta SL, Wood J, Hicks JB. A plant DNA mini preparation: Version II. Plant Molecular Biology Reporter. 1983;1(4)19-21.

49. Doyle JJ and Doyle JL. A rapid isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin. 1987;19(1)11-15.

50. Benbouza H, Baudoin JP, Mergeai G. Amélioration de la méthode d'extraction d'ADN au CTAB appliquée aux feuilles de cotonnier. Biotechnology, Agronomy and Society and Environment. 2006;10: 73-76.

51. Agbangla C, Ahanhanzo C, Tostain S, Dansi A, Daïnou O. Evaluation de la diversité génétique par RAPD d'un échantillon de *Dioscorea alata* d'une région du Bénin, la sous-préfecture de Savè. Jounal de la Recherche Scientifique de l'Université de Lomé. 2002;6:197-202.

52. Mongkolporn O, Kadkol GP, Pang ECK, Taylor WJ. Identification of RAPD markers linked to recessive genes conferring siliqua shatter resistance in *Brassica rapa*. Plant Breeding. 2003;122:479-484.

53. Imazio S, Labra M, Grassi1 F, Scienza A, Failla O. Chloroplast microsatellites to investigate the origin of grapevine. Genetic Resources and Crop Evolution. 2006;53:1003-1011.

54. Wills DM & Burke JM. Chloroplast DNA variation confirms a single origin of domesticated sunflower (*Helianthus annuus* L.). Heredity. 2006;97:403-4.

55. Chaïr H, Perrier X, Agbangla C, Marchand JL, Daïnou O, Noyer JL. Use of cpSSRs for the characterisation of yam phylogeny in Benin. Genome. 2005;48:674-684.

56. Decroocq V, Hagen LS, Favé M-G, Eyquard J-P, Pierronnet A. Microsatellite markers in the hexaploid *Prunus domestica* species and parentage lineage of three European plum cultivars using nuclear and chloroplast simple-sequence repeats. Molecular Breeding. 2004;13:135-42.

57. Grivet D. Phylogéographie et évolution moléculaire comparée d'arbres forestiers à l'aide des marqueurs chloroplastiques. Sciences agricoles. Université Henri Poincaré - Nancy 1, 2002. Français. ⟨NNT : 2002NAN10002⟩.

58. Ndoyé K. La gestion des ressources génétiques au Sénégal : application au complexe d'espèces *Acacia nilotica* (L.) willd.. Dakar : UCAD, Thèse 3e cycle : Biologie végétale. Option Ressources génétiques et amélioration des plantes : Université Cheikh Anta Diop. 2001 ; 121 p.

59. Oueslati Bahri A. Diversité et origine génétique des espèces de la sous famille des Aurantioideae et décryptage des structures interspécifiques des génomes des agrumes modernes. Thèse. Université de Tunis, Tunisie. 2017 ; 200p.

60. Andrianoelina Andrianaivo O. Diversité génétique, physiologie de reproduction et étude d'impact de la fragmentation sur *Dalbergia monticola* de la forêt orientale de Madagascar. Thèse. Université d'Antananarivo, Madagascar. 2009; 140p.

61. Hutchinson J, Dalziel JM, & Keay RWJ. Flora of west tropical Africa (2nd ed., Vol. I). London: The Whitefriars Press. 1958.

62. Amahowé IO, Djossa AB, Adomou AC, Kabré AT & Sinsin B. Vegetation structure and tree diversity in habitats exploited by elephants in Benin (West Africa). Journal of Applied Biosciences. 2012;58:4223– 4233.

63. Luo CW, Li K, Chen Y, Sun YY. Floral display and breeding system of *Jatropha curcas* L. For Stud China. 2007;9:114–119.

64. Hasibuzzaman, AS. M., Islam, A. A., Miah, M. G., & Hasan, M. (2020). Phylogeographic diversity and population structure of *Carica papaya* L. revealed through nuclear microsatellites. Brazilian Journal of Botany. 2020;43(1)147-154. doi.org/10.1007/s4041 5-020-00594 -8.

65. Ov I, Espinosa-García FJ, Nuntilde JS, Salvador-Figueroa M. State of the art of genetic diversity research in *Jatropha curcas*. Scientifique Research and Essays. 2011;6:1709–1719.

66. Deng Y, Liu T, Xie Y, Wei Y, Xie Z, Shi Y, Deng X. High Genetic Diversity and Low Differentiation in *Michelia shiluensis*, an Endangered Magnolia Species in South China. Forests. 2020;11(4)469; doi: 10.3390/f11040469.

67. Fontaine C, Lovett PN, Sanou H, Maley J, Bouvet J-M. Genetic diversity of the shea tree (*Vitellaria paradoxa* C.F. Gaertn), detected by RAPD and chloroplast microsatellite markers. Heredity. 2004;93:639-648.