

**Spatial scale patterns of genetic diversity and gene flow in populations of sweet detar  
(*Detariummicrocarpum*Guill. &Perr.;Fabaceae)**

**Abstract**

The main objective of this study is to investigate the patterns of genetic diversity and phylogenetic relationships within populations of *Detariummicrocarpum*(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 clustershaplotypes 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.

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

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### Abbreviations

AMOVA: Analysis of molecular variance

cpDNA: Chloroplastic DNA

CTAB: CetylTriammonium Bromide

GPS: Global Position System

MATAB: Mixed Alkyl Trimethyl Ammonium Bromide

PCR: Polymerase Chain Reaction

PIC: Polymorphism Information Content

SSR: Simple sequence repeat

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### Introduction

*Detarium microcarpum* Guill. & Perr. (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 has 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 (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], etc. 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. Hence the need for the establishment of 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

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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 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.

UNDER PEER REVIEW

## 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 GPS and those belonging to the same phytogeographic district are considered to be from the same population (Table 2).

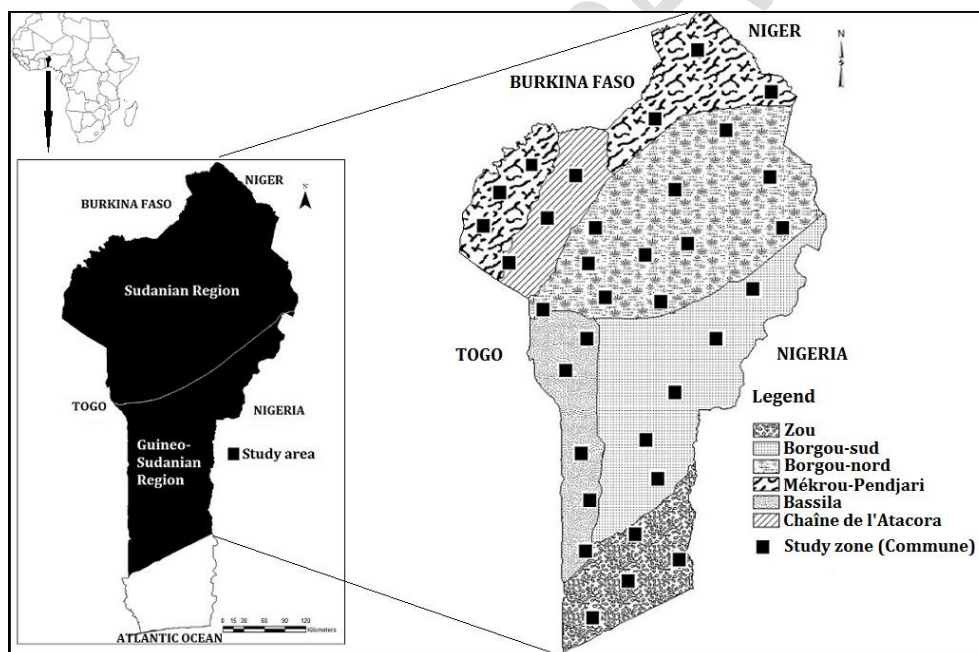


Fig. 1: Map of Benin showing the location of sampling areas.

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

Phytogeographic district	Climatic zone	Rainfall regime	Rainfall	Major soil types	Major plant formation
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(mm)					
Bassila	Guineo-Sudanian	Tendency to unimodal	Min: 1100 Max: 1300	Ferrallitic soils with concretions and breastplates	Semi-deciduous forest, woodland, and riparian forest
Zou			Min: 1100 Max: 1200	Ferruginous soils on crystalline rocks	Dry forest, woodland, and riparian forest
South Borgou					
North Borgou					
Atacora Chain	Sudanian	Unimodal (1 rainy season)	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'	Atacorachain (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'	

<b>BaCo117</b>	9°56.263'	1°31.824'		CaNa122	10°10.972'	1°24.796'	
<b>BaDj164</b>	9°41.364'	1°35.958'		CaNa124	10°21.805'	1°21.151'	
<b>BaDj167</b>	9°41.877'	1°30.041'		CaNa145	10°25.105'	1°21.715'	
<b>BaDj169</b>	9°40.455'	1°41.667'		CaTo126	10°28.387'	1°22.130'	
<b>BaDj170</b>	9°39.701'	1°42.187'		CaTo130	10°29.293'	1°22.543'	
<b>BnBe63</b>	9°50.575'	2°59.107'		MpBa100	11°15.101'	2°38.385'	
<b>BnBe64</b>	10°00.937'	2°40.142'		MpBa979	11°18.339'	2°31.929'	
<b>BnGo78</b>	10°54.550'	2°51.288'		MpCo138	10°28.515'	1°02.096'	
<b>BnKa111</b>	10°40.175'	3°34.961'		MpCo139	10°30.047'	1°00.575'	
<b>BnKa112</b>	11°38.037'	3°34.894'		MpCo142	10°33.703'	0°59.623'	
<b>BnKa113</b>	10°28.458'	3°32.159'		MpMa143	11°44.326'	3°13.268'	
<b>BnKa81</b>	11°05.820'	2°59.740'	NorthBorgou (NB)	MpMa144	11°41.418'	3°12.490'	Mekrou-Pendjari (MP)
<b>BnKa83</b>	11°05.552'	3°00.054'		MpMa85	11°40.966'	3°12.099'	
<b>BnPe161</b>	10°14.913'	1°53.586'		MpMa88	11°39.939'	3°11.739'	
<b>BnSe105</b>	10°57.214'	3°25.377'		MpMa91	11°32.960'	3°07.250'	
<b>BnSe108</b>	10°50.773'	3°38.025'		MpMa94	11°30.346'	3°06.257'	
<b>BnSi70</b>	10°16.518'	2°27.544'		MpTa132	10°37.822'	1°17.967'	
<b>BnSi73</b>	10°21.275'	2°23.450'		MpTa135	10°39.378'	1°14.613'	
<b>BsNd54</b>	9°44.539'	2°41.790'		ZoDa28	7°48.352	2°10.954'	
<b>BsNd56</b>	9°44.550'	2°41.753'		ZoDa29	7°59.986	2°26.874'	
<b>BsNi57</b>	9°54.184'	2°55.997'		ZoDa30	7°57.564	2°28.336'	
<b>BsNi58</b>	9°49.231'	2°59.327'	South Borgou (SB)	ZoDj1	7°30.848	2°05.411'	Zou (ZO)
<b>BsOu35</b>	8°28.530'	2°26.392'		ZoDj2	7°31.037	2°05.459'	
<b>BsPa51</b>	9°20.206'	2°36.978'		ZoDj3	7°31.511	2°04.566'	
<b>BsPa52</b>	9°20.132'	2°34.349'		ZoDj6	7°31.546	2°04.507'	
<b>BsPe61</b>	9°50.008'	2°59.302'		ZoDj13	7°29.755	1°59.643'	

<b>BsTc41</b>	8°23.426'	2°29.306'	ZoDj15	7°29.771	1°59.635'
<b>BsTc42</b>	8°52.915'	2°34.969'	ZoDj23	7°29.520	1°59.889'
<b>BsTc47</b>	8°54.157'	2°35.799'	ZoGl31	8°01.233	1°59.083'
<b>BsTc49</b>	8°55.208'	2°35.397'	ZoS1187	7°55.353	2°00.811'
<b>BsTc50</b>	9°02.079'	2°36.925'	ZoS34	8°28.551	2°26.384'

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### 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	Dellaporta et al. [48]	Doyle & Doyle [49]	Agbangla et al. [51] ( <i>Dioscoreaspp.</i> )	Benbouza et al. [50] ( <i>Gossypiumspp.</i> )	Adjé et al. [29] ( <i>Ananas cormosus</i> )
<b>Composition</b>					
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	-
$\beta$ mercaptoethanol (%)	0.07	0.5	-	5	0.1
Charcoal (mg.g <sup>-1</sup> )	-	-	-	10-15	-

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<b>SDS</b>	20%	-	-	-	-
<b>Incubation time at 65°C (min)</b>	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:  $[DNA] = A_{260} \times DF \times 50 \mu\text{g/ml}$ , where [DNA] is the DNA concentration, A260 is the Absorbance at 260 nm, DF = Dilution Factor; 50  $\mu\text{g/ml}$  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 /  $\mu\text{L}$  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 (*Nicotianatabacum* 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  $\mu\text{L}$ ) of PCR master mix composed of 200  $\mu\text{M}$  dNTP; 0.2  $\mu\text{M}$  of each primer (forward and reverse); 1.25  $\mu\text{M}$  of MgCl<sub>2</sub>; 0.1U/ $\mu\text{L}$  of TaqDNA-polymerase; 5  $\mu\text{L}$  of 5X Tris-HCl buffer and 9.25  $\mu\text{L}$  of

ultrapure water were added to 3  $\mu$ L of DNA (approximately 3ng/  $\mu$ L) 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. <i>tabacum</i> (pb)	PIC	Na
Ntcp_8	trnG intron	T11	F: ATATTGTTTTAGCTCGGTGG R: TCATTCGGCTCCTTTATG	55	251	0.52	3
Ntcp_9	trnG/trnR intergenicregion	T10	F: CTCCAAGCTAACGATGC R: CTGTCCTATCCATTAGACAA TG	55	237	0.43	2
Ntcp_3 3	rpoA exon	T10	F: TGGCTGTTATTCAAAAAGGTC R: CATGATAAATTGGCTAAACT CA	60	149	-	1
Ntcp_3 6	rps19/rpl2 intergenicregion	T14	F: GTAGTAAATAGGAGAGAAA ATAG R: ATGATACATAGTGCGATACA G	50	125	0.59	3
Ntcp_3 7	rrn5/trnRintergenicregion	A13	F: TTCCGAGGTGTGAAGTGG R: CAGGATGATAAAAAGCTTA ACAC	55	143	0.65	3
Ntcp_3 9	trnR/rrn5 intergenicregion	T13	F: GTCACAATTGGGGTTTTGAA TA	60	156	0.64	3

			R: GACGATACTGTAGGGGAGG TC				
<b>Ccmp<sub>2</sub></b>	5' to trnSintergenic region	A11	F: GATCCCGGACGTAATCCTG R: ATCGTACCGAGGGTTCGAAT	60	189	0.73	7

Ccmp: consensus chloroplast microsatellite primers; Ntcp: *Nicotianatabacum* 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<sub>3</sub>) 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 ( $H_e$ ), Observed heterozygosity ( $H_o$ ), 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 ( $M_p$ ) over the total number of markers ( $M_t$ ):  $P = M_p/M_t \times 100$ . 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:  $PIC = 1 - \sum f_i^2$  where  $f_i$ , 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:  $A = (1/k) \sum_{n=1}^k n_i$  where  $k$  is the total number of loci and  $n_i$  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 ( $A_e$ ) was computed by the formula:  $A_e = 1 / \sum (f_i)^2$  where  $f_i$  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 ( $H_e$ ) or genetic diversity of Nei was calculated by locus according to the formula:  $H_e = 1 - \sum (f_i)^2$ , where  $f_i$  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 cpDNA of *D. microcarpum* [40]. Rate of observed heterozygosity ( $H_o$ ) was calculated from the measured frequency of heterozygotes by:

$H_o = N_h/(N_t)$  where  $N_h$  is the number of heterozygous individuals and  $N_t$  the total number of individuals [39]. By comparing these two indices ( $H_e$  and  $H_o$ ), 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:  $F_{IS} = 1 - (H_o / H_e)$ . 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  $H_S$  is the average  $H_e$  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

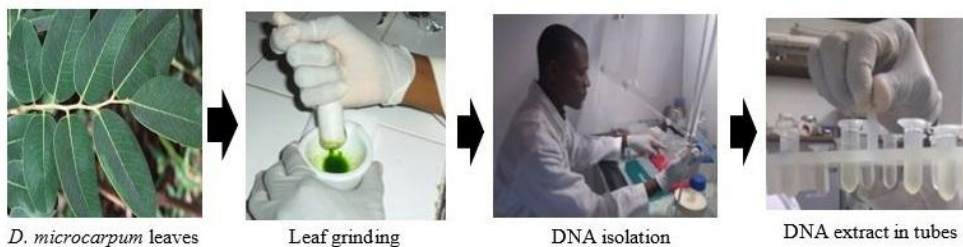
### Genomic DNA isolation buffer of the new protocol

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 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%  $\beta$ -Mercaptoethanol and 0.5% Proteinase K (added after sterilization and just before use in a hood).

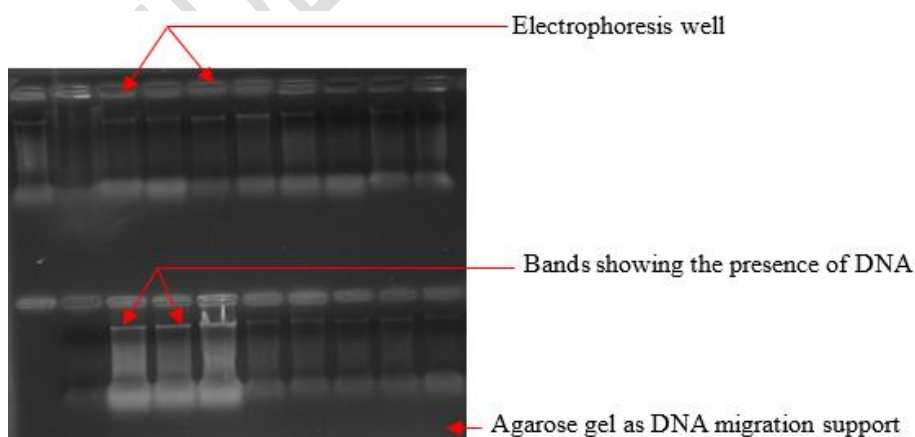
### New protocol for genomic DNA isolation from *D. microcarpum*

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%  $\beta$ -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  $\mu$ L 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  $\mu$ L 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  $\mu$ L 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  $-20^{\circ}\text{C}$  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.



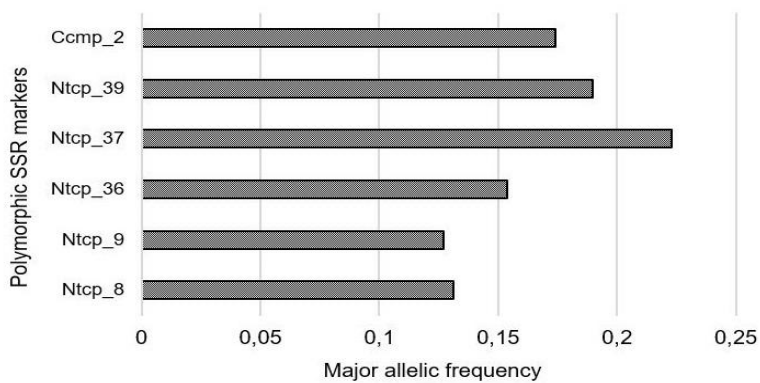
**Fig. 2:** Some steps in the classic process of isolation *Detariummicrocarpum* genomic DNA.



**Fig. 3:** Analytical electrophoresis profiles on agarose gel of genomic DNA from some *Detariummicrocarpum* 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 <sub>IS</sub>
				Ho	He	
AC	13	2.00	2.053	0.603	0.513	-0.175
BA	13	1.42	1.769	0.230	0.435	0.471
MP	13	1.85	1.776	0.297	0.437	0.321
NB	13	2.42	2.092	0.425	0.522	0.186
SB	13	2.85	2.531	0.455	0.605	0.248
ZO	13	1.71	1.923	0.324	0.480	0.325
<b>Mean</b>	<b>13</b>	<b>2.04</b>	<b>2.024</b>	<b>0.389</b>	<b>0.498</b>	<b>0.229</b>

N: sampling collection size per population, A: allelic richness, Ae: effective allele, Ho: observed heterozygosity, He: expected heterozygosity, F<sub>IS</sub>: 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<sub>ST</sub>) is 0.024. Phylogenetic analysis revealed that the N<sub>ST</sub> value was significantly higher than the G<sub>ST</sub> value (N<sub>ST</sub> = 0.452; G<sub>ST</sub> = 0.190; P < 0.05). A relatively low h<sub>d</sub>

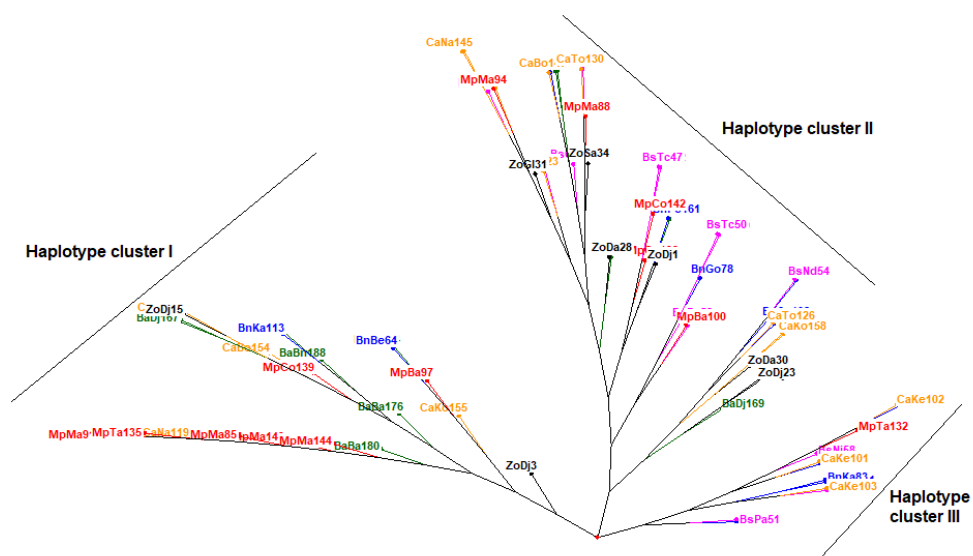
Source of Variation	Degree of Freedom	Sum of Squares	Variance Components	Percentage of variation	p Value
Among populations	5	50.175	3.246	45.80%	< 0.01
Within populations	14	48.307	0.584	17.35%	< 0.01

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haplotypediversity 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.

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**Fig. 5:** Neighbor-joining phylogenetic tree showing haplotypes clusters from *Detariummicrocarpum* 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 *Ceratoniasiliqua*[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]. This is 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 Cetyltrimmonium Bromide (CTAB) and that of Agbangla et al. [51] using Mixed Alkyl Trimethyl 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  $\beta$ -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], Chair 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 Borgouphytogeographic 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 Chainphytogeographic 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  $P_p < 0.05$ , 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 & 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

We found 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 phylogeographic districts. There is an existence of gene flow within *D. microcarpum* populations in phylogeographic districts. Our 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.

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## 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 phylogeographic 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 DISCLAIMER:

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.

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