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Genetic diversity of *Dacryodes buettneri* (Engl.) H.J. Lam (Burseraceae), a timber tree in Central Africa

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Abstract: In order to contribute to the management of plant genetic resources in tropical Africa, a study of the genetic diversity of *Dacryodes buettneri*, a forest species exploited in Central Africa as timber, was carried out in Gabon. The nuclear DNA extracted from 170 trees distributed in six populations was amplified using five microsatellite markers. This amplification revealed that the genetic diversity within population is relatively important: the mean number of alleles per locus A=4.43±0.93, the percentage of polymorphism P=76% and expected heterozygosis H_{exp}= 0.35±0.06. The mean of inbreeding coefficient, F is equal to 0.25±0.08. The values of F-statistics showed that the genetic variability of populations taken separately is less than genetic variability in the meta-population (*Fis*=38% and *Fit* = 40%). The mean genetic distance between populations is low *Fst*=0.08, indicating much of genetic diversity within population. The gene flow is *Nm*=2.88. The genetic diversity of *D. buettneri*, showing an important inbreeding coefficient, is related to the reproductive system and mode of seed dispersal. The results contribute to valorization and conservation of wild phytogenetic resources for sustainable use.

Keywords: genetic diversity, Dacryodes buettneri*, microsatellites, Central Africa, sustainable use*

Introduction

Dacryodes buettneri (Engl.) H.J. Lam belongs to Burseraceae family. It is a species of the upper canopy of evergreen forests, hitherto undomesticated (Bouroubou

Bouroubou, 1994). It can reach 50 m in height (White and Abernethy, 1996) and up to 150 cm in diameter (Aubreville, 1962; White and Abernethy, 1996). It is characterized by its leaflets hairy, reddish-brown below (Figure 1a), leaf character that distinguishes it from other species of *Dacryodes*. The outer bark of its trunk is golden yellow and peeling in long and wide blades (Figure 1b). Its main use is exploitation as timber (Wilks and Issembe, 2000). *D. buettneri* (trade name: ozigo) is counted among the top five species exploited in Gabon and supplies the domestic and international markets. It is sold in combination with okoume, *Aucoumea klaineana* (Todou and Doumenge, 2008). It is also an important fruit in Gabon. Its fruits are purplish at maturity, edible, resembling those of *D. edulis* (G. Don) H.J. Lam (Vivien and Faure, 1985), but they are smaller, less valued by local people and they are not yet traded. Uses for medicinal purposes are much localized in villages (Raponda-Walker and Sillans, 1961).

The importance of *D. buettneri* is well-known by local people and timber traders. It undergoes genetic erosion because of anarchic agriculture and the forest exploitation like all forest species in equatorial Africa in spite of its importance. In Cameroon, *D. buettneri* is reported threatened due to the pressure exerted by the exploitation of the forest and the relatively small number of trees in populations (Todou and Doumenge, 2008; Onana, 2008). The regeneration of forests is mainly by seeds from trees in situ. It depends on the maintenance of genetic diversity, especially to cope with unpredictable climate change or to establish a program of forest exploitation. *D. buettneri* is counted among the species which their genetic organizations are unknown. The lack of interest in genetic studies on this species was guided this study because this is a useful species, but it is highly threatened by both

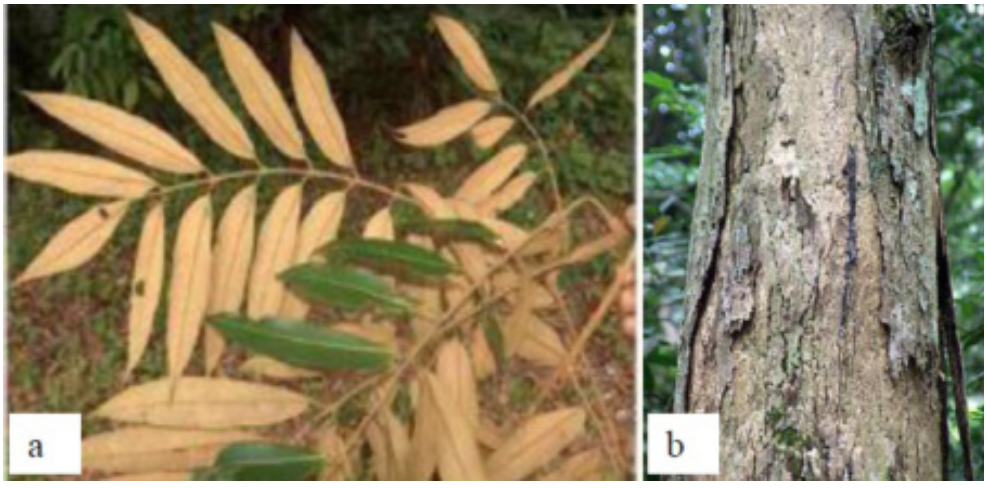

Figure 1 - Details of morphological structures of D. buettneri*. a: leaves, b: trunk.*

climatic conditions and high anthropogenic pressures.

The present study focuses on the genetic diversity in natural populations of *D. buettneri* in Gabon through the amplification of neutral genetic markers. The objective is to determine the parameters of population genetics of the species: the intra-population and inter-population structure and genetic diversities. Thus, different populations can be compared with each other and it will be highlighted with those characteristics.

Materials and methods

Sampling of populations

Six populations of Dacryodes buettneri were sampled in Gabon (Figure 2). In total, 170 leaflets or fragments of cambium were collected on each tree. They were wrapped in filter paper and stored in sealed plastic bags with cilica gel. The main criteria that guided the sampling populations were diverse climatic and ecological complexity. Each sample has a collection's number, the name of the species, some morphological descriptions and the GPS coordinates of the individual collected (longitude, latitude and elevation). In most cases which individuals are little distance, a name given to the site where the sample was collected, the coordinates of a central point is took by equating to a population.

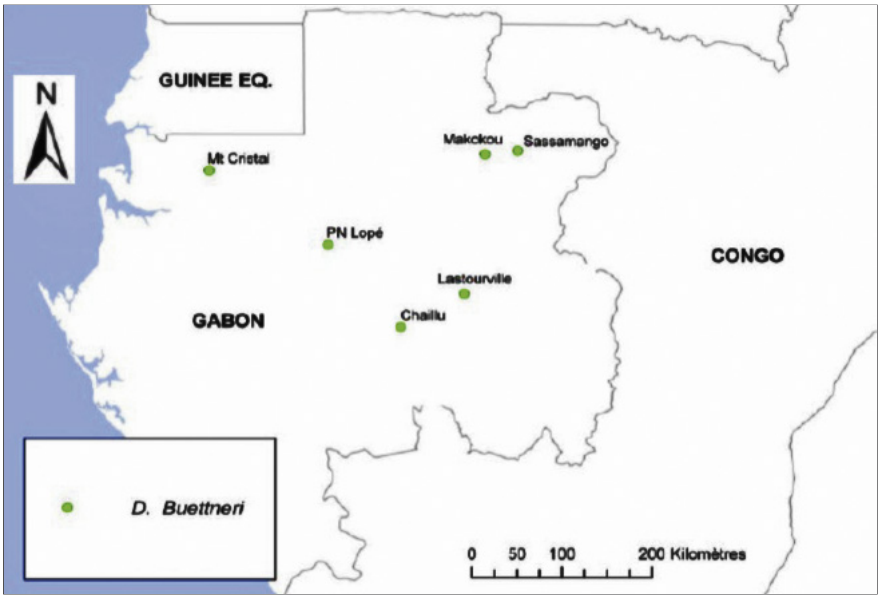

Figure 2 - Distribution of sampled populations in the study site.

Extraction of nDNA, PCR and genotyping

Fragments of 10 mg of dried plant material were finely ground with a ball of tungsten by stirring in a mill (Retsch MM301). The nuclear DNA (nDNA) was extracted following the protocol of DNeasy 96 Plant Kit (Qiagen, Hilden, Germany 2006). The nDNA of each individual was amplified using microsatellite loci developed on D. edulis by Benoit *et al.* (2011). The multiplex PCR kit (Qiagen, Hilden, Germany 2006) was done following the protocol provided for a volume of 10 µL: 5 µL of 2 × Qiagen Multiplex Master Mix; 1 µL of primer mix (mix of complementary primers, 2 µM); 1 µL of H_2O and 2 µL of nDNA extracted. Amplifications of nDNA were performed in thermal cyclers (Eppendorf Mastercycler gradient) in the laboratory of Genetic Markers Common Service of CEFE Montpellier. The primer pairs were associated in both groups (CB09, LD06 and CG11, LB12, CE09) and 1 µL of the PCR product diluted (1/100) was added to 15 µL of deionized formamide and 0.2 µL of LIZ (size marker labeled with a fluorochrome). Genotyping was done on the ABI PRISM capillary sequencer 16 by IFR 119 (University of Montpellier 2, France).

Processing and analysis of data

The results collected electronically by the sequencer were processed with GeneMapper 3.5 software (Applied Biosystems, Foster City, California, USA). The software determined the number of alleles and assigned alleles size (number of base pairs). We used the Hardy-Weinberg expectations to test if populations are in panmictic equilibrium. The populations' genetic parameters were calculated by Genetix 4.05 software (Belkhir 1994-2004). Two patterns of parameters of the genetic diversity were calculated and interpreted.

Parameters reflecting intra-population diversity

The mean number of alleles per locus (A) is the ratio of total of alleles and the number of studied loci. A was calculated as follows:

$$\mathrm{A} = \frac{\sum_{\mathrm{i=1}}^{\mathrm{n}} \mathrm{ai}}{\mathrm{L}} \qquad A = \frac{\sum_{i=1}^{L} a_i}{L}$$

a_i is the number of alleles to locus i and L is the number of studied loci

The percentage of polymorphic loci (P) is the number of polymorphic loci in relation to number of studied loci. It was calculated as follows:

$$P = \frac{x}{m}$$

x is the number of polymorphic loci and *m* is the total number of loci.

The mean percentage of polymorphic loci for one species is the average over different studied populations.

The heterozygosis expresses the probability that two that a randomly selected individual had different alleles at a locus. It is defined as H_{exp} (expected heterozygosis) and it is equivalent to the theoretical heterozygosis. In one population in equilibrium, the frequency of each genotype is the product of its allele frequencies (pi). The expected heterozygosis at locus k was calculated as follows:

$$H_{exp} = 1 - \sum_{i=1}^{ak} p^2 ik$$

ak is the number of alleles to locus *k*

The observed heterozygosis (H_{obs}) to locus k was calculated as follows:

$$H_{obsk} = \sum_{ij=1}^{ak} pik \ (i \neq j)$$

pij is the estimate of frequencies of genotype *ij* to locus *k*

The expected heterozygosis (H_{exp}) and observed heterozygosis (H_{obs}) of one species are the arithmetic mean of H_{exp} or H_{obs} over studied populations.

The inbreeding coefficient or fixation index (F) expresses the deviation of Hardy-Weinberg equilibrium. It was calculated as follows:

$$F = 1 - \frac{H_{obs}}{H_{exp}}$$

F varies from -1 to +1. If *F* negative, there is heterozygote excess in the studied population and if *F* positive, there is heterozygote deficit in the studied population.

Parameters reflecting inter-population diversity

F-statistics express the pattern of genetic diversity in divided meta-population as sub-populations (Weir and Cockerham, 1984).

Fst provides a measure of the genetic differentiation among populations. It is the proportion of the total genetic diversity (~ heterozygosity) that separates the populations or the inbreeding coefficient within subpopulations, relative to the total.

The value of *Fst* was calculated as follows*:*

$$F_{ST} = 1 - \frac{(1 - F_{IT})}{(1 - F_{IS})}$$

Where, Fis and *Fit* measure the correlation between random gametes of same individual respectively in sub-populations and meta-population. *Fis* expresses the

local heterozygote deficit in populations in relation to Hardy-Weinberg equilibrium, while *Fit* expresses the global heterozygote deficit in the meta-population taken on the whole. *Fst* expresses the correlation between two random gametes in two different populations. It measures the level of genetic differentiation between these populations.

According to Wright (1965), if $0 < Fst < 0.05$, the genetic differentiation is considered as feeble; if $0.05 < Fst < 0.15$, the genetic differentiation is considered as moderate; if $0.15 < Fst < 0.25$, the genetic differentiation is considered as important and if $Fst > 0.25$, the genetic differentiation is considered as very important.

The gene flow (*Nm*) expresses the degree of exchange of genes between populations and where *m* is the rate of migration and *N* is effective number of individuals in the population.

The genetic differentiation between populations is limited by the gene flow between these populations. *Nm* was calculated as follows:

$$Nm = \frac{1 - Fst}{4 * Fst}$$

Results

Allele's sizes per locus

Five microsatellite loci were isolated from *D. edulis* by Benoit (2011) and used in this study for the facility of their amplification on *D. buettneri*.

The study of genetic diversity of *D. buettneri* was based on the variation of heterozygosis of individuals by selected microsatellites markers. The allele's sizes expressed in number of pairs of bases (pb) were variable. Allele's size ranged from 167 to 192 pb and 146 to 164 pb for CB09 and CE09, respectively, while it ranged from 175 to 183 pb for CG11 (Table 1 and Annex 1).

Frequency of alleles

Loci studied have globally many alleles (Table 2). CE09 (17 different alleles) and CB09 (10 different alleles) are the most rich loci in alleles while CG11 is the less rich (4 different alleles). The total number of alleles is 43 for all loci in six studied populations. Several loci have frequency of allele equal to 0 (Annex 1). Two loci have several fixed alleles of which the allelic frequencies equal 1. The allele 181 of GC11 is fixed in Lastourville population. The frequencies of this allele vary from 0.87 to 1 in all populations. The allele 154 of LD06 locus is fixed in PN Lopé population and Crystal Mt population.

Mean number of alleles per locus and per population

The mean number of alleles per population is variable within populations. The

Table 1 - Characteristics of the loci pairs used to study genetic diversity of D. buettneri.

Markers names	Allele size range (pb)	Repeat motif	Primer sequences (5'– 3')
CB09	163 – 200	(GA) 10	F: CAGTGGTAAAGGTAGGATGG R: GGTTTGTGTTGGTTGTTTTC
LD06	152 – 175	(GA) 10	F: CATGTGCATCTTTTGATCTC R: TAGAGGGGAAAGGTAGAAGG
CG11	170 – 185	(CT) 8	F: CACACGATCAAGAAAGACC R: CCCGTAATGACTCTCTAATTG
LB12	205 – 230	(GA) 14	F: AGACCACAAGAACCTTTGC R: GGCCACTTAATTTGATTTTG
CE09	125 – 168	(GA) 16	F: GTAGCTGGGAGGAGATCAGG R: ACAGGCCCCAAAAGCAC

Table 2 - Total numbers of alleles per population of D. buettneri *and per locus.*

Populations	CB09	CE09	CG11	LB12	LD09	A
Chaillu	9	12	3	5	5	6,8±3,63
Lastourville	6	5	1	2	3	3,4±2,07
Makoukou	5	4	2	2	2	3±1,41
Sassamango	9	9	2	2	2	4,8±3,83
Mt Cristal	9	5	2	2	1	3,8±3,27
PN Lope	9	9	2	3	1	4,8±3,89
Mean	7,83±1,83	7,33±3,14	2±0,63	2,66±1,21	2,33±1,50	4,43±0,93

A = Mean number of alleles per population (± standard error).

total mean for all loci is 4.43 ± 0.93 alleles per population. The mean number of alleles per locus ranged from 3±1.41 in Makoukou population to 6.8±3.63 in Chaillu population (Table 2). CE09 and CB09 are the most polymorphic with respectively 17 and 10 alleles for all populations. CG11, LB12 and LD09 are less polymorphic loci with 7 alleles or less.

Polymorphism of populations

The studied populations have 76% of mean percentage of polymorphism (Table 3). The polymorphism varies from 60% in Sassamango and PN Lopé populations to 100% in Chaillu population. Other populations have 80% of polymorphism.

Heterozygosis

The observed heterozygosis (H_{obs}) varies from 0.21 in Sassamango population to 0.34 in Chaillu population. The mean observed heterozygosis in all populations is 0.26±0.04. The means of expected heterozygosis (H_{exp}) are between 0.27 in

Table 3 - Indices of intra-population diversity estimated for D. buettneri.

POPULATIONS	N	P	HEXP	HOBS	F
CHAILLU	36	1	0,47	0,34	0,27
LASTOURVILLE	23	0,8	0,36	0,23	0,36
MAKOUKOU	27	0,8	0,36	0,25	0,30
SASSAMANGO	28	0,6	0,27	0,21	0,22
MT CRISTAL	25	0,8	0,32	0,27	0,15
PN LOPE	31	0,6	0,34	0,28	0,17
MEAN	28±4,63	0,76±0,15	0,35±0,06	0,26±0,04	0,25±0,08

N = Number of individuals, P = Percentage of polymorphism, Hobs = observed heterozygosis, Hesp = expected heterozygosis and F = inbreeding coefficient

Sassamango population and 0.47 in Chaillu population. The mean of expected heterozygosis of all populations is 0.35±0.06 (Table 3).

Inbreeding coefficient

The inbreeding coefficient F (or fixation index) reflects the deviation from Hardy-Weinberg equilibrium. The six populations studied have positive inbreeding coefficients indicating the heterozygote deficit in the populations. These coefficients vary from 0.15 (in Mt Crystal population) to 0.36 (in Lastourville population). The mean is 0.25±0.08 (Table 3).

F-statistics and genetic distances between populations

The genetic diversity of sub-populations taken separately, Fis = 0.38, is close to the genetic diversity of the meta-population, Fit = 0.40 (Table 4). However, the intra-population diversity in populations taken separately is lower than the intra-population diversity in meta-population taken on the whole. The mean of genetic differentiation between populations is globally moderate (Fst = 0.08). The mean value of Fst indicates that more genetic diversity of *D. buettneri* is located in population (intra-population diversity). Only 8% of genetic diversity is at inter-population while up to 92% of genetic diversity is at intra-population.

The Table 5 indicates that the genetic distances between populations are low (Fst < 0.05) or moderate (0.05 < Fst < 0.15). Genetic distances vary from 0.02 to 0.10. Makokou population is the most genetically distant from other populations. These values indicate that populations are genetically similar and belong to the same genetic group. The gene flows vary from 1.50 (between Mt Cristal and Makokou) to 14.30

Table 4 - F-statistics and gene flow (averages of 6 populations).

Parameters	Fis	Fit	Fst	Nm
Mean values	0,38	0,40	0,08	2,87

Table 5 - Gene flow matrix (above the diagonal) and genetic distance (Fst) (below the diagonal).

Populations	Chaillu	Lastourville	Makokou	Sassamango	Monts de Cristal	PN Lope
Chaillu	****	10,47	4,57	2,60	5,78	4,92
Lastourville	0,02	****	2,17	1,56	4,62	2,24
Makokou	0,05	0,10	****	1,81	1,50	1,68
Sassamango	0,08	0,14	0,12	****	4,94	6,83
Monts de Cristal	0,04	0,05	0,14	0,05	****	14,30
PN Lope	0,05	0,10	0,13	0,03	0,01	****

* significant moderate genetic distance between populations

(between Mt Cristal and PN Lopé). The values of genetic distances increase when those of gene flows decrease.

Discussion and conclusion

The mean value of heterozygosis of *Dacryodes buettneri* (H_{exp} = 0.35±0.06 and A = 4.43±0.93) is lower than those estimated in *Entandrophragma cylindricum*, a timber species also exploited in equatorial Africa and in *Vitex fischeri* and *V. keniensis*. Our results are similar with mean heterozygosis estimated parameters in *Ficus insipida* and those generated in *Aucoumea klaineana*. In our study, *D. buettneri* showed high genetic diversity compared to most tropical Sudanian species that have been studied by enzymatic electrophoresis as *Acacia Senegal* and *Vitellaria paradoxa* (Table 6).

The massif forests of Mt Cristal, PN Lopé and Chaillu are in the domain of the CARPE program. This program is a vast regional network having a basis for the implementation of conservation priority. The massif forest of Makoukou is close to National Park of Irvindo. The study of genetic parameters of six strategic studied populations of *D. buettneri* helped to highlight the level of diversity of species populations and to compare them. Chaillu population is particularly most genetically diverse (mean number of alleles per locus A = 6.8±3.63; percentage of polymorphism P = 100% and the rate of expected heterozygosis Hexp = 0.47) followed by Lastourville population and Makoukou population. Sassamango population is the less genetically diverse (A = 4.8±3.83; Hexp = 0.27 and P = 60%). All of these massif forests are able to be in program of conservation.

Table 6 - Heterozygosis of some species.

SPECIES	Hexp	A	REFERENCES
Entandrophragma cylindricum	0.85	18.5	GARCIA *et al.* (2004)
Vitex fischeri and *V. keniensi*	0.97	5.1	AHENDA (1992)
Ficus insipida	0.35	-	VIGNES *et al.* (2006)
Aucoumea klaineana	0.46	5.2	BORN *et al.* (2006)
Acacia senegal	0.18	-	CHEVALIER *et al.* (1994)
Vitellaria paradoxa	0.21	-	LOVETT AND HAQ (2000)

H_{exp} = expected heterozygosis, A = mean number of alleles

The inbreeding coefficient reflects an important deficit of heterozygotes in studied populations. In mean, F = 0.25±0.08, is comparable to the inbreeding coefficient of *Parkia biglobosa*, F = 0.24 (Sibidou Sina, 2006). The value of the inbreeding coefficient of *D. buettneri* is close to that of much forest species. Indeed, *D. buettneri* is dioecious (Hecketsweiler, 1992) but there are some individuals of this species which have both male and female flowers, and individuals which have hermaphrodite flowers. This last situation combined with the natural distribution of individuals could favor the autogamy causing a heterozygote deficit.

Genetic distances or genetic differentiation, which in mean value is Fst = 0.08, are considered as moderate. This value is comparable with other tropical forest species. The mean genetic distance of *D. buettneri* is higher than that of *Vitellaria paradoxa*, a species of the Sudano-Sahelian zone, which Fst values are 0.0124, 0.026 and 0.047 (Lovett and Haq, 2000; Cardi, 2005; Sanou, 2005) and that of *Cecropia obtusifolia* which Fst = 0.029 (Alvarez-Buylla and Garay, 1994). However, the mean genetic distance of *D. buettneri* is less than that of tropical species (Fst = 0.119) (Loveless, 1992). It is also lower than that reported for *Faidherbia albida*, a Sudano-Sahelian species, which Fst = 0.123 (Joly 1992). Some species have a higher genetic differentiation (*Elaeis guineensis*, Fst = 0.301, (Hayati, 2004), *Acacia auriculiformis*, Gst = 0.270 (Wicknesmari and Norwati, 1993).

Genetic diversity of *D. buettneri* supports the reproductive system and mode of seed dispersal. All mature trees of *D. buettneri* are not flowering simultaneously in a year and they are not involved in reproduction (Hecketsweiler, 1992; Bourobou Bourobou, 1994). Irregular participation of adult individuals in reproduction can favor some genotypes. Besides, trees flowering earlier in the season are preferentially pollinated by other trees flowering earlier and it is the same for individuals flowering later. The inbreeding could increase the heterozygote deficit. This mode of reproduction seems still exceptional for many species of tropical forests and our results are in consonance with this observation. In addition, 92% of the genetic diversity is

due to intra-population variation (Fst = 0.08). Indeed, most forest species have vast and continuous distribution areas and the seeds are widely dispersed. The conclusion is therefore that *D. buettneri* is differentiated within their populations but less between populations as other tropical species (Hamrick 1993).

Seeds dispersal also plays an important role in the heterozygote deficit. Indeed, *D. buettneri* is barochore, fruits are eaten and dispersed by arboreal monkeys and large frugivorous birds (White and Abernethy, 1996; White 1999; Hecketsweiler, 1992). The role of wild animals (monkeys, squirrels and birds) in the seeds dispersal of *D. buettneri* could be important. It is a species of forest areas whose fruits are eaten by these animals (Todou and Doumenge, 2008). Thus, Hamrick *et al.* (1993) suggest that plant species which fruits are eaten by animals are highly genetically variable within populations, but not between populations.

The values of genetic distances between populations of *D. buettneri* are lower than those of *Vitex kiniensis* and *Vitex fischeri* (Ahenda, 1999). Indeed, the values of gene flow are relatively large. The mean gene flow within *D. buettneri* is Nm = 2.87, which shows that almost three individuals from one population can have microsatellite sequences similarly to individuals of another population during one generation. The gene flow between populations is favored by short geographic distance and outcrossing. The largest value of gene flow was observed between PN Lopé population and Mt Crystal population (Nm = 14.30) and the lowest value was noted between Mt Cristal population and Makoukou population.

The in situ conservation of forest genetic resources is a conservation strategy that requires the support of politicians and sponsors like Non Governmental Organizations. It must be integrated into the overall framework of sustainable forest management to be effective. However, the practical aspects of how to implement the current in situ conservation need knowledge of the genetic diversity in natural populations. This study highlights a relatively high level of genetic diversity, mainly from intra-population in *D. buettneri.* The mode of reproduction and seed dispersal mode are the main factors that explain the current genetic structure of *D. buettneri.* Also, its origin and distribution focused on Gabon corroborate the low genetic distances found between the populations analyzed. Considering the genetic variation existing within the species is an important step in the conservation of genetic resources of *D. buettneri* that populations are increasingly threatened by timber exploitation.

Annex 1 - Allelic frequencies of population of D. buettneri.

Loci	Alleles	Chaillu	Lastourville	Makokou	Sassamango	Mt Cristal	PN Lope
CB09	167	0.129	0.296	0.058	0.036	0.140	0.113
	169	0	0	0.039	0.018	0.040	0.081
	176	0.014	0.023	0	0.214	0.020	0.048
	177	0.129	0.136	0.519	0.482	0.260	0.274
	179	0.029	0	0	0	0	0.065
	181	0.114	0	0	0.018	0.100	0.210
	185	0.214	0.341	0.154	0.054	0.280	0.097
	187	0.286	0.159	0.231	0.107	0.100	0.097
	189	0.071	0	0	0.018	0.020	0.016
	192	0.014	0.046	0	0.054	0.040	0
CE09	126	0.042	0	0	0	0	0
	127	0.014	0.022	0	0.036	0.020	0.161
	128	0.125	0.044	0.222	0.036	0.080	0.113
	130	0.653	0.891	0.648	0.732	0.840	0.645
	132	0	0	0	0.018	0	0
	134	0	0	0	0.018	0	0.032
	135	0	0.022	0	0.018	0	0
	137	0.014	0	0	0	0	0
	138	0.014	0	0	0	0	0
	141	0.014	0	0.111	0.018	0	0.048
	143	0.028	0	0	0	0	0
	145	0.014	0	0	0	0	0
	150	0.014	0	0	0	0.040	0
	155	0.028	0	0	0	0	0
	156	0	0	0	0.018	0	0
	158	0.042	0	0	0	0	0
	159	0	0.022	0.019	0.107	0.020	0
CG11	175	0	0	0.056	0	0	0
	180	0.111	0	0	0.036	0.100	0.048
	181	0.875	1	0.944	0.964	0.900	0.952
	183	0.014	0	0	0	0	0
LB12	210	0.014	0	0	0	0	0
	219	0.819	0.727	0.982	0.929	0.800	0.855
	220	0.139	0.273	0.019	0.071	0.200	0.145
	221	0.014	0	0	0	0	0
	230	0.014	0	0	0	0	0
LD06	146	0	0.044	0	0	0	0
	154	0.700	0.674	0.556	0.982	1	1
	155	0.257	0.283	0.444	0	0	0
	156	0	0	0	0.018	0	0
	159	0.014	0	0	0	0	0
	160	0.014	0	0	0	0	0
	164	0.014	0	0	0	0	0

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