Available Online at ESci Journals
Journal of Plant Breeding and Genetics
ISSN: 2305-297X (Online), 2308-121X (Print)
http://www.escijournals.net/JPBG

NUCLEAR DNA CONTENT ANALYSIS OF FOUR CULTIVATED SPECIES OF YAMS (DIOSCOREA SPP.) FROM CAMEROON

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A B S T R A C T

Yam (*Dioscorea* spp.) is an important food source in Africa, but diseases and storage pests hinder the African farmers to achieve high yields during the harvest. One important limitation to the genetic breeding of yam is the relatively unknown ploidy level variation within and among species. The objective of this study was to determine the nuclear DNA content of 59 accessions representing four cultivated *Dioscorea* species collected from three regions of Cameroon (Adamawa, Centre and Southwest) using flow cytometry with propidium iodide staining. Our findings suggested the variation of the genome size both within and among the yams species. Nuclear DNA content (mean 2C-value) in studied yam collection ranging from 0.72 ± 0.013 pg in *D. dumetorum* to 2.801 ± 0.068 pg in *D. cayenensis.* The accessions could be divided into four different categories according to their, nuclear DNA content suggestive of four different ploidy levels. Ploidy variation was observed within all species with the exception of *D. dumetorum* that is likely diploid. This study contributes to a better understanding of the genome characteristics of yam species from Cameroon and may help to the genetic improvement of this important crop in the future.

Keywords: *Dioscorea*, nuclear DNA content, Polyploidy, flow cytometry, genome.

INTRODUCTION

Yams are monocots of the genus *Dioscorea* that belongs to the *Dioscoreaceae* family. The genus *Dioscorea* consists of about 600 species that are mainly distributed in subtropical or tropical areas of Africa, America, Asia and Polynesia (Coursey 1967; Ayensu and Coursey 1972). In Cameroon, the yam is third after cassava and cocoyam/taro according to the volume of plant roots and tubers produced (FIDA, 2003). Usually consumed boiled, yam contributes to food security in this region of Africa. The main *Dioscorea* species in Cameroon include *Dioscorea alata* L. (greater yam or water yam), the Guinea yam complex *Dioscorea cayenensis* Lam. (yellow) / *Dioscorea rotundata* Poir. (white), and *Dioscorea dumetorum* (Kunth) Pax (bitter yam). All these cultivated species are susceptible to pests (nematodes) and diseases (Orkwor *et al.,* 1998). In order to develop better genotypes locally adapted and tolerant to pests and diseases, plant breeders need a wide range of genetic diversity (Dansi *et al.,* 2000) to search for genes of interest and generate efficient genotypes from hybridization. However, variation in ploidy level among accessions can hamper such hybridization efforts by giving poorly fertile hybrids due to the cytological irregularities. Consequently, a good knowledge of the ploidy level of yam species is imperative for the establishment of successful breeding programs. Furthermore, it has become important to know the genome structure for sequencing projects because the scale and cost of sequencing is based on their genome size (Dolezel and Greilhuber 2010).

Flow cytometry is increasingly used to determine or screen the ploidy level in this genus (Egesi *et al.,* 2002; Dansi *et al.,* 2005; Obidiegwu *et al.,* 2009a; Nemorin *et al.,* 2013). A recent cytogenetic study perform in *D. alata* has yet again confirmed the coherence of flow cytometric indicators of ploidy level in yams (Norman

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et al., 2012). This method, which deduces ploidy from the quantity of nuclear DNA (2C=2*n*), has many advantages: it is rapid, non-destructive (one sample can be prepared from a few milligrams of leaf tissue), sensitive and convenient. In addition, flow cytometry does not require dividing cells and can detect both mixoploidy and potentially aneuploidy (Galbraith *et a1.,* 1983; Marie and Brown 1993; Dolezel, 1997). Diploid, tetraploid and octoploids individuals have been identified in Cameroon accessions of the *D. cayenensis-D. rotundata* complex (Dansi *et al.,* 2001a). Otherwise, nothing has been reported concerning the nuclear DNA content of Cameroon yam species. The aim of this work was thus to determine the nuclear DNA content and deduce potential ploidy level of the four major cultivated Cameroon yams. This characterization of available germplasm should strengthen our understanding of the pan-African resources, favouring development of effective protocols for the production, exchange and breeding of this important crop.

MATERIALS AND METHODS

Plant material: Tuber accessions representing four *Dioscorea* species (*D. alata, D. cayenensis*, *D. dumetorum* and *D. rotundata*) were collected from three regions (Adamawa, Centre and Southwest) of Cameroon (Fig.1). These tubers were planted in polyethylene bags containing sterile black soil. After germination, the plants were transferred and then maintained as a field collection or as plantlets in test tubes incubated in growth room under 80 µmol.m^{-2}.s^{-1} light provided by cool white fluorescent tube lamps (Mazda) at a photoperiod of 16 h at 26 ± 1°C. Taxonomic identification was achieved at the National Herbarium of Cameroon using descriptions from different treatments (Baker 1898; De wildeman 1914; Knuth 1924). Diploid *Solanum lycopersicum* L."Montfavet 63/5" (2C=2*x*=1.99 pg; Lepers-Andrzejewski *et al.,* 2011) or *Petunia hybrida* L. PxPC6 (2*n*=2*x*=14, 2C=2.85 pg; Marie and Brown 1993) were used as internal standards for flow cytometry. *Petunia hybrida* was used with *D. alata* because the 2C nuclei of tomato and yam were too close.

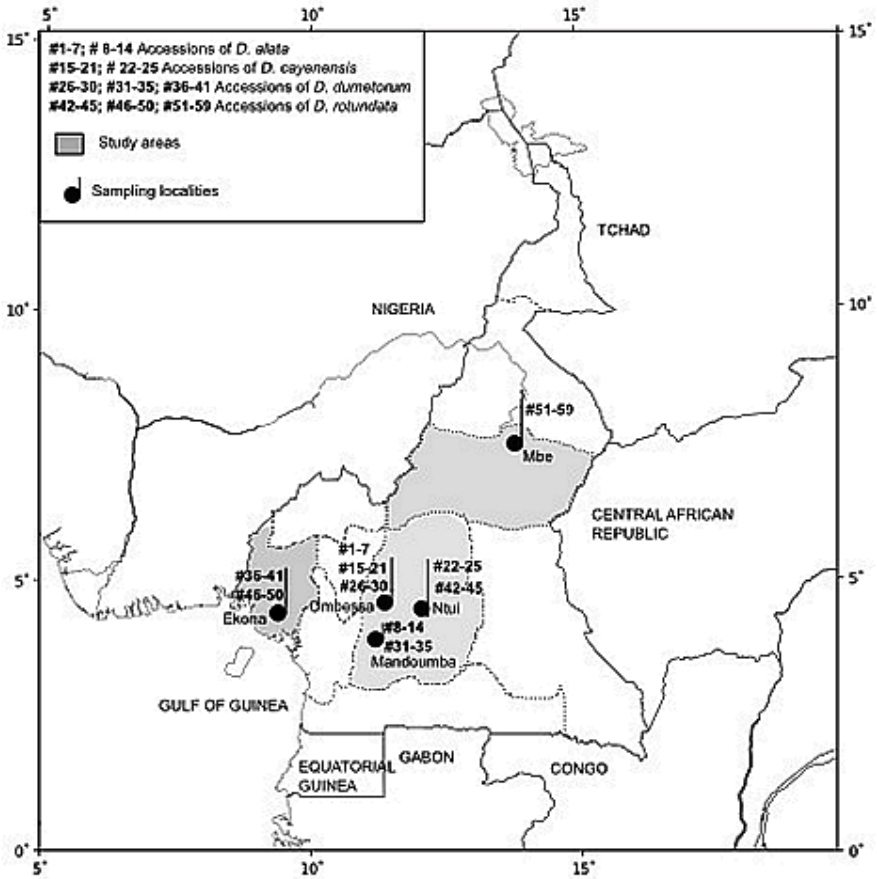

Figure 1. Map of Cameroon indicating the five localities and the three regions where we collected the 59 accessions of four *Dioscorea* yams species.

Preparation of nuclei for cytometry: Leaves were collected from 59 plants in the field, bagged, transported at cold temperature and kept in a refrigerator at 6°C for a maximum period of 4 days until analysis. For each accession, nuclei were isolated by a method adapted from Galbraith et al. (1983): 50 mg of yam leaf together combined with 20 mg of leaf from the internal standard was chopped with a razor blade in a Petri dish containing 1 mL of cold lysis buffer modified by adding 5 mM sodium metabisulfite and 1% (w/v) polyvinylpyrrolidone 10000. In the case of *D. cayenensis* and *D. rotundata*, β-mercaptoethanol at 5 $\mu L.mL^{-1}$ was added instead to cope with the negative effect of phenolic compounds released from the stored leaves upon chopping. Occasionally, the stem was taken as tissue, but due to endoreduplication (Brown et al. 1991). The histograms must be carefully examined as the critical 2C peak can be of minor frequency. Nuclear suspensions were filtered through 50-µm nylon (CellTrics, Partec) and stained with 50 $\mu g\ mL^{-1}$ propidium iodide (PI: Sigma-Aldrich, France). RNase, at a concentration of 50 $mg\ mL^{-1}$ (Roche, France), was added to the suspension to avoid staining of double-stranded RNA by propidium iodide. After 5 min incubation on ice, nuclear suspensions were ready for cytometry.

Flow cytometry measurements: The nuclear suspensions were analyzed with a flow cytometer (at CNRS using a CyFlowSL, Partec Münster) equipped with a 30 mW 532 nm laser. PI fluorescence emitted from nuclei was collected through a 560 nm long-pass filter and a 630 nm band-pass filter. For each sample, fluorescence data was acquired from at least 5000 nuclei. Three independent replications were done and histograms with coefficient of variation (CV) above 5% were rejected. The 2C nuclear DNA content was calculated by multiplying the known DNA content of the internal standard (*S. lycopersicum* or *P. hybrida*) by the quotient between the 2C peak positions of *Dioscorea* and internal standard in the histogram of fluorescence intensity.

Ploidy level inference and statistical analyses: To group DNA content into putative ploidy level with no a priori knowledge, we used a clustering approach by kmeans. To select the optimum number of groups, we classified the DNA content into x = 1, 2, 3, …, 10 groups and for each classification the percentage of variance explained among groups was estimated. The optimal number of groups was considered to be the number above which the addition of another group did not increase the among-group variance by more than 5%. We then attributed putative ploidy level to these groups based on Zounfjihékpom et al. (1993). The latter authors have observed that *D. rotundata*, tetraploids (4x), hexaploids (6x) and octaploids (8x) had 2C DNA content of 1.24, 1.77 and 2.59 pg, respectively. The putative ploidy levels were partitioned geographically for the four species; a Fisher exact test was performed**.** The kmeans clustering and Fisher's exact test were performed using the software R version 3.0.2 (http://www.R-project.org/.).

RESULTS

Our results show that the 2C DNA content ranged from 0.717 pg in *D. dumetorum* to 2.801 pg in *D. cayenensis* (Table 1, 2). The kmeans clustering showed an optimal clustering with four groups of 2C DNA content as the addition of a fifth group only increased the among groups variance by 1% (Fig. 2). The cluster centers in terms of DNA contents were estimated to be 0.72, 1.33, 1.84, and 2.8 pg. These findings are supported by the density plot of the 2C DNA content that clearly shows four distinct peaks (Fig. 3).

Table 1. List of 59 yam accessions from Cameroon with their nuclear DNA content and their putative ploidy level. The fluorescence intensities for each accession and its internal standard are indicated (AU, arbitrary channel units). The fluorescence intensity is indicated by p for *P. hybrida* and by s for *S. lycopersicum*.

Species	Accession number	Geographical origin	Accession 2C Intensity (AU)	Standard 2C intensity (AU)	2C DNA (pg)	Tentative Ploidy Level (x)
	1	Ombessa	274	423^{p}	1.846	6
	2	Ombessa	289	443^{p}	1.859	6
	3	Ombessa	291	443^{p}	1.872	6
	4	Ombessa	294	444^{p}	1.887	6
	5	Ombessa	291	442^{p}	1.876	6
	6	Ombessa	290	441^{p}	1.874	6
D. alata	7	Ombessa	292	442^{p}	1.883	6
	8	Mandoumba	296	444^{s}	1.900	6
	9	Mandoumba	287	436^{s}	1.876	6

Continue…

	10	Mandoumba	167	402[s]	1.184	4
	11	Mandoumba	174	421[s]	1.178	4
	12	Mandoumba	169	416[s]	1.158	4
	13	Mandoumba	172	427[s]	1.148	4
	14	Mandoumba	172	425[s]	1.153	4
	15	Ombessa	284	410[s]	1.378	4
	16	Ombessa	207	302[s]	1.364	4
	17	Ombessa	195	277[s]	1.428	4
	18	Ombessa	216	301[s]	1.435	4
D. cayenensis	19	Ombessa	235	326[s]	1.404	4
	20	Ombessa	19,7	14,5[s]	2.700	8
	21	Ombessa	429	298[s]	2.865	8
	22	Ntui	400	289[s]	2.754	8
	23	Ntui	389	273[s]	2.836	8
	24	Ntui	19,7	14,1[s]	2.780	8
	25	Ntui	469	325[s]	2.872	8
	26	Ombessa	156	433[s]	0.717	2
	27	Ombessa	159	445[s]	0.711	2
	28	Ombessa	167	473[s]	0.703	2
	29	Ombessa	171	469[s]	0.726	2
	30	Ombessa	170	465[s]	0.728	2
	31	Mandoumba	161	448[s]	0.715	2
D. dumetorum	32	Mandoumba	160	435[s]	0.732	2
	33	Mandoumba	173	463[s]	0.744	2
	34	Mandoumba	172	479[s]	0.715	2
	35	Mandoumba	161	461[s]	0.695	2
	36	Ekona	166	464[s]	0.712	2
	37	Ekona	162	448[s]	0.720	2
	38	Ekona	155	432[s]	0.714	2
	39	Ekona	159	456[s]	0.694	2
	40	Ekona	159	434[s]	0.729	2
	41	Ekona	166	460[s]	0.718	2
	42	Ntui	250	384[s]	1.296	4
	43	Ntui	259	391[s]	1.318	4
	44	Ntui	260	385[s]	1.344	4
	45	Ntui	294	396[s]	1.477	4
	46	Ekona	289	409[s]	1.406	4
	47	Ekona	19,7	14,9[s]	1.316	4
	48	Ekona	283	408[s]	1.380	4
	49	Ekona	272	407[s]	1.330	4
D. rotundata	50	Ekona	252	382[s]	1.313	4
	51	Mbé	244	380[s]	1.278	4
	52	Mbé	8,8	13,3[s]	1.317	4
	53	Mbé	277	394[s]	1.399	4
	54	Mbé	243	386[s]	1.253	4
	55	Mbé	267	375[s]	1.417	4
	56	Mbé	337	429[s]	1.563	6
	57	Mbé	205	333[s]	1.755	6
	58	Mbé	193	342[s]	1.608	6
	59	Mbé	188	339[s]	1.581	6

Table 2. Yam species (*Dioscorea*) from Cameroon with their mean nuclear DNA in pg and in Mega base pairs (Mbp)(1pg = 0.978 Mbp; Dolezel et al. 2003) as well as their putative ploidy level. The uniformity of groups is expressed as Coefficients of Variation (CV).

Species	2C-value mean ± SD (pg)	1C-value (Mbp)	1Cx-value	CV(%)	Putative Ploidy level
D. alata	1.164 ± 0.016	569	0.291	1.36	4x
	1.875 ± 0.016	916	0.312	0.83	6x
D. cayenensis	1. 0.027	685	0.350	1.95	6x
	2.801 ± 0.068	1369	0.350	2.45	8x
D. dumetorum	0.717 ± 0.013	350	0.358	1.83	2x
D. rotundata	1.346 ± 0.062	658	0.338	4.59	4x
	1.627 ± 0.087	795	0.271	5.36	6x

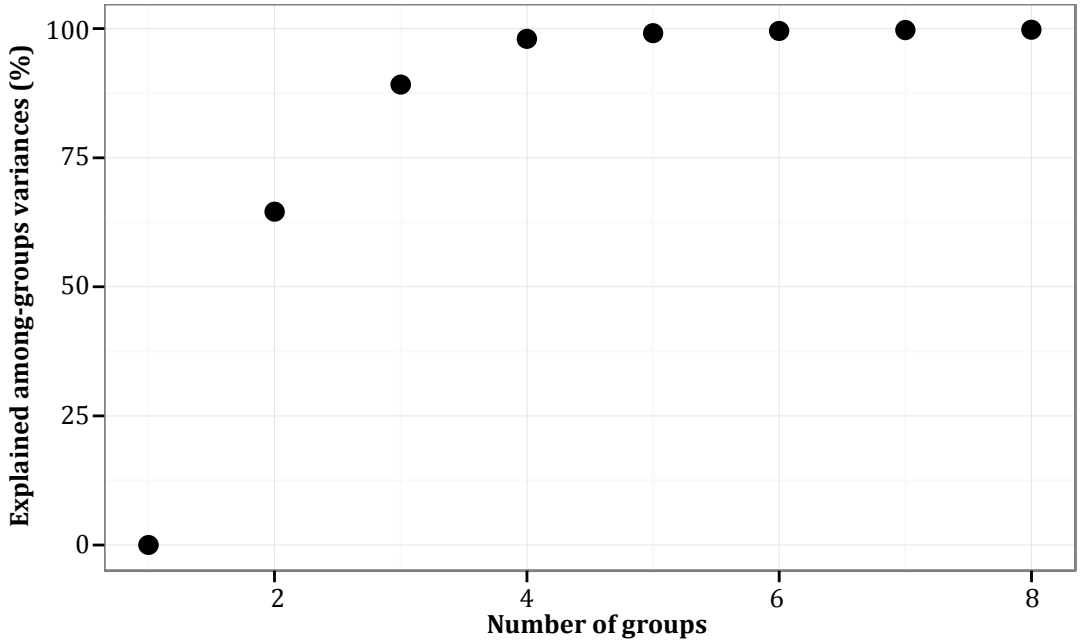

Figure 2. Percentage of among group variance for different number of groups in kmeans clustering.

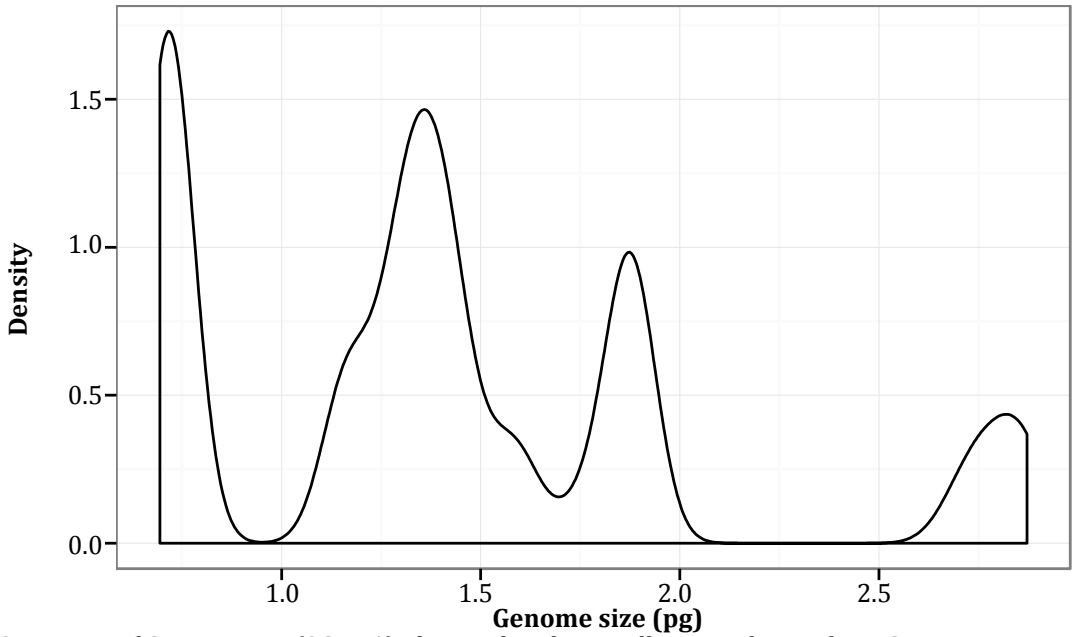

Figure 3. Density of Genome size (2CDNA) observed within a collection of yams from Cameroon.

Using the known nuclear DNA content per ploidy level for *D. rotundata*, these four clusters were tentatively assumed to represent diploids, tetraploids, hexaploids and octoploids individuals. All *D. dumetorum* accessions were classified in a single cluster for DNA content, and evidence suggest these are all diploids. None of the other species were found to contain diploids. Putative tetraploids were found in *D. alata, D. cayenensis*, and *D. rotundata*, putative hexaploids were found in *D. alata* and *D. rotundata*, and putative octoploids were only observed in *D. cayenensis* (Table 1 & 2; Fig4). No correlation was found between the geographical origin of accessions and putative ploidy level (Fisher's exact test; $p > 0.05$ after Holm Sidak correction).

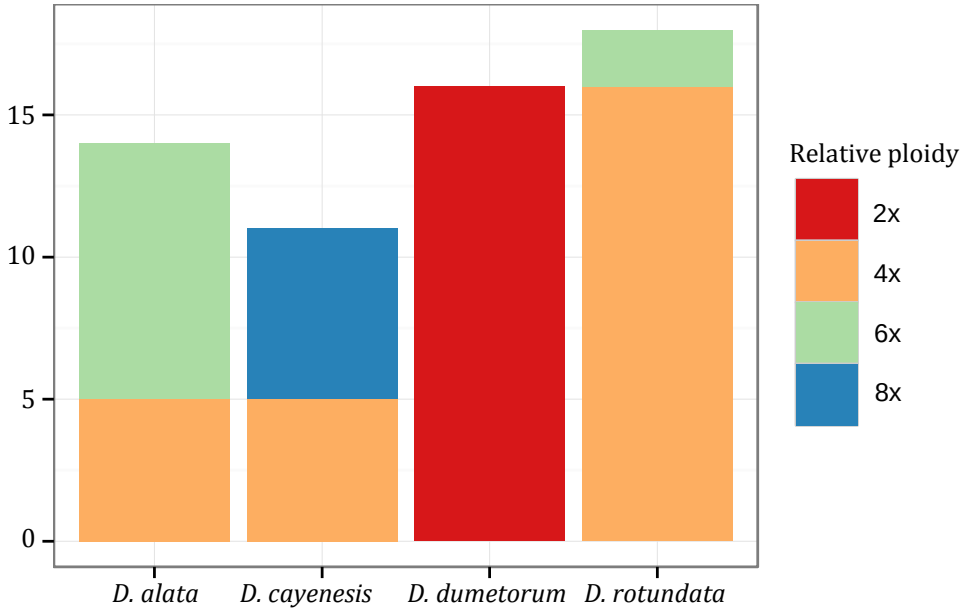

Figure 4. Tentative ploidy levels within each yam species (*Dioscorea* spp.) from Cameroon with their relative frequencies indicated by the numbers of accessions.

DISCUSSION

In our study, the holoploid genome size (1C-value) ranged from 0.358 pg in *D. rotundata* to 1.400 pg in *D. dumetorum*. These are very small genomes (about twice that of *Arabidopsis thaliana*; Marie and Brown 1993) according to Leitch's classification (Leitch *et al.,* 2005). Our results concur with those of Hamon *et al.* (1992) and Obidiegwu *et al.* (2009b) that reported 2C-values ranges of 0.88-2.88 pg and 0.702-2.573 pg in *Dioscorea*, respectively. Our results also agree with these previous studies that suggested the diploid 2C content in *D. dumetorum* was approximately 0.7 pg. The mean 2C-value observed for *D. dumetorum* in the present study is the lowest recorded in the genus to date. The 2C value of 1.164 pg for our putative tetraploid *D. alata* is close to that reported by Hamon *et al*. (1992). These authors also reported 0.885 pg for a supposed 2n=3x=30, in this case 1Cx is 0.291, as indeed we obtain in Table 2.

The variation in the genome size observed within and among species of yams could be attributed to several mechanisms. Variation in noncoding regions such as numbers of transposable elements (TEs) has been shown to be a source of genome size augmentation (Bennetzen, 2000). Genome size can also be reduced, and this can be caused, amongst other causes, by the deletion of the non-coding or duplicated DNA sequences (Bennetzen, 2005). In some case, real variation within species can be explained by the differential presence of supernumerary B chromosomes (Gregory et al. 2005). The variation observed in this study for DNA content appears to be mostly affected by ploidy level variation (Otto et al. 2007). The very distinct clusters of DNA content clearly argue for this alternative, even if other mechanisms could explain variation within these ploidy levels.

In summary, four ploidy levels have been inferred in the studied panel: diploid in *D. dumetorum*; tetraploid in *D. alata, D. cayenensis* and *D rotundata;* hexaploid in *D. alata* and *D. rotundata*; octoploid in *D. cayenensis*. Overall, tetraploid individuals are the most abundant, as

Essad (1984) reported for the genus and as Egesi *et al.* (2002) reported for *D. alata* from Chad and Puerto Rico. Unlike Arnau *et al.* (2009) and Muthamia *et al.* (2014), we did not find any triploid and pentaploid in *D. alata* from Cameroun. The octoploids were also absent among the accessions of *D. alata* analysed. Yet, this does not mean that they are absent from *D. alata* because they may be found elsewhere. For instance, octoploids have been reported in *D. alata* from the germplasm collection of the International Institute of Tropical Agriculture, Ibadan-Nigeria (Obidiegwu *et al.,* 2010).

Our findings have also shown that *D. dumetorum* is potentially only diploid in Cameroun. This supports an earlier study of Obidiegwu *et al.* (2009b) that indicated the predominance of diploidy in this species. Unlike Sharma and De (1956) and Obidiegwu *et al.* (2009b), we have not identified any triploid individuals among our accessions. Both tetraploid and hexaploid individuals were observed in *D. rotundata* (white yams) in our study. Hexaploids, which have been reported previously in Cameroon (Dansi *et al.,* 2001), were found only in the Adamawa region. According to Dansi et al. (2001), hexaploid cultivars would be hybrids potentially derived from a natural cross between octoploid cultivars of *D. cayenensis* and tetraploid cultivars of *D. rotundata.* These hexaploids individuals could thus form a *D. cayenensis* - *D. rotundata* complex. Our study could not confirm this hypothesis; molecular markers would be required for such a test. Yet, it is interesting that no *D. cayenensis* were sampled from the Adamaoua region. And on the contrary, at the Ntui site in the Centre region, tetraploid *D. rotundata* and octoploid *D. cayenensis* were sympatric, but no putative hybrid hexaploids were observed. But our sampling size is too small for these observations to be statistically relevant. The tetraploidy of *D. rotundata* has been previously reported by several authors (Hamon et al, 1993; Dansi et al. 2000; Mingnouma et al. 2002; Obidiegwu et al. 2009a*;* Muthamia et al. 2014). Yet, contrary to these authors and to our results, Dainou et al.2000 had suggested that *D. rotundada* is diploid. Scarcelli *et al.* (2005) had approved this statement using segregation pattern of isozyme loci and microsatellite markers. More recently, a study on the diversity and evolution of guinea yams base on genotyping and cytometry has underline the importance to associate the molecular data to the flow cytometry method in order to confirm the real ploidy level of the yams species, because the yams species haven't the same evolutionary history (Girma et *al.* 2014). In view of this latest study, the investigations cytogenetic and molecular as the genotyping based on the new generation of sequencing will have to be performed in the future on these yams species, particularly in *D. alata* and *D. dumetorum*, in order to provide a definitive conclusion on their ploidy level.

This investigation is a preliminary step towards the establishment of breeding programs for yam in Cameroon, and evaluation of novel germplasm for the whole continent. It provides the informations on the variation of nuclear DNA content and the natural variation of ploidy within and between yam species in Cameroon for which such data is rare, with the exception of Dansi *et al.* (2001a) using the IITA accessions in Ibadan. Polyploidy must be take into account in future amelioration programs. It is an important factor in the choice of plant material and breeding methods. Natural or cultivated populations and subsequent formal accessions need to be rigorously characterised for their 2C value and their ploidy, by comparison with permanently established reference plants of known cytogenetic formulae.

ACKNOWLEDGEMENTS

The authors thank Association Francaise de cytométrie and L'OREAL-UNESCO for their grants in the achievement of this project. Thank also to Samba, Belinga Hans, Oumar, André Mbairanodji for their assistance during collecting of plant material.

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