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RESEARCH ARTICLE

Ploidy levels of *Dioscorea alata* L. germplasm determined by flow cytometry

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Abstract *Dioscorea alata* L. is a highly important crop, widely distributed in the humid and semi-humid tropics. Flow cytometry was used to determine the ploidy levels of 74 *D. alata* genotypes collected mainly from West African countries. Sixty three of the genotypes were found to be tetraploid, one was hexaploid and ten were octoploid. The high percentage of tetraploids together with the small percentage of hexaploid individuals and the absence of diploid individuals gives us some more clues on the possible origin of these species. No association between ploidy level and place of cultivation was found for the tested material. The obtained results represent important knowledge for enhancing the breeding methodologies and optimize germplasm management of this species. It also offers further insights to the phylogeny and evolution of *Dioscorea* species.

Keywords Breeding · *Dioscorea alata* · Flow cytometry · Genetic resources · Germplasm · Ploidy level

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Introduction

Dioscorea alata L. (greater yam) is the most widely distributed species of the genus *Dioscorea* L. (Dioscoreaceae) in the humid and semi-humid tropics. The origin of this species is still a matter of debate but it is generally accepted that it belongs to the Enantiophyllum section of Southeast Asia. The underground tubers of *D. alata* are used almost exclusively for human consumption in Africa, the Caribbean, and especially Melanesia where it has considerable social and cultural importance (Coursey

1972, 1976). The greater yam is superior to most edible yam species in terms of yield potential under adverse weather conditions, ease of propagation, early vigour for weed suppression and tuber storage (Hahn et al. 1987). This species is mainly composed of ancient, vegetatively propagated clones, thus, the continuous accumulation of disadvantageous somatic mutations or viral infections has led to the clonal degeneration of many genotypes of this economically important crop.

Despite *D. alata* has often been regarded in the past as a sterile crop (due to seldom seed set) (Coursey 1967), recent efforts by the International Institute of Tropical Agriculture (IITA, Nigeria) and the Central Tuber Crop Research Institute (CTCRI, India) have shown that natural and artificial pollination, and subsequent production of fertile seeds, can be achieved under optimized culture and management practices (Egesi et al. 2001). Basic knowledge about the genetic resources of yams is fundamental for the implementation of successful breeding programs. The basic chromosome number of *D. alata* is $x = 10$, and many polyploid clones have been already documented ($2n = 4x$, $6x$, $8x$) (Martin and Ortiz 1966; Zoundjihékpon et al. 1990; Egesi et al. 2002). Therefore, cytological irregularities that lead to erratic flowering and reproductive behaviour (e.g., non viable or genetically unstable progeny) are expected in this crop (Egesi et al. 2002). Recently, a core collection of *D. alata* germplasm held in trust by IITA was developed and characterized (Mahalakshmi et al. 2007). However, the lack of information on ploidy level of this core set limits its utility in future breeding programs.

In plants, ploidy estimation is traditionally performed by chromosome counting on microscope slides. This method has already been used in *Dioscorea* spp. with success (Martin and Ortiz 1966; Zoundjihékpon et al. 1990), but since their chromosomes are very small and "dot-like," counting is laborious and fraught of inaccuracies. Due to these drawbacks, the application of this method for large-scale screening of the ploidy level of yam accessions has been continuously reduced in favour of more reliable methodologies, such as flow cytometry (FCM). The major advantages of this technique are the convenience, precision and rapidity of the analysis, the requirement of a small amount of tissue, and the ability to analyse large populations of nuclei (Doležel et al. 2007). Its application in the analysis of the ploidy level and/or estimation of genome size in *Dioscorea* has already been demonstrated to be very successful and convenient (Hamon et al. 1992; Egesi et al. 2002; Obidiegwu et al. 2009).

The main goal of this study was to determine the ploidy level of the *D. alata* core accessions held in trust by the IITA using flow cytometry. It is our believe that a better understanding of the reproductive biology through studies on ploidy level of the *D. alata* core set will lead to a more precise and efficient use of the genetic diversity for the purpose of a systematic hybridization with alleviated incompatibility problems, which may enhance the breeding methodologies and optimize germplasm management of this crop.

Materials and methods

Plant material

The plant material consisted of 74 accessions of *D. alata* L. (landraces and breeder's lines) from nine countries, mostly in West Africa (Table 1). These accessions have been previously identified as the species core set (Mahalakshmi et al. 2007). Plants were cultivated in 30 cm size pots filled with sterilized top soil and maintained in a screenhouse at the International Institute of Tropical Agriculture (IITA, Ibadan, Nigeria).

Sample preparation for ploidy analyses

Young leaves were collected from individual plants, moistened in paper, bagged, transported at cold temperature, and kept in a refrigerator for a maximum period of 5 days until analysis. In most cases, only healthy looking leaves were used for ploidy level analysis.

Nuclear suspensions were obtained after chopping 40–50 mg of leaf tissue per genotype of greater yam with a sharp razor blade in a glass Petri dish containing 1 ml LB01 lysis buffer (5 mM TRIS, 2 mM Na_2EDTA, 0.5 mM spermine·4HCl, 80 mM KCl, 20 mM NaCl, 15 mM β-mercaptoethanol, 0.1% (v/v) Triton X-100, pH 7.5; Doležel et al. 1989). The inclusion of an anti-oxidant, β-mercaptoethanol, in the buffer composition was essential to circumvent

Table 1 Origin and ploidy level of the 74 accessions of greater yam (*Dioscorea alata* L.) analysed in this study using flow cytometry

Genebank accession code	Cultivar or local name	Country of origin	Ploidy level
TDa 3919	Doutierou	Benin	4*x*
TDa 3944	Agba	Benin	4*x*
TDa 3898	Sanse	Benin	4*x*
TDa 1178	BE 136	Benin	4*x*
TDa 3922	Souanrou	Benin	4*x*
TDa 1190	BE 114	Benin	4*x*
TDa 1240	BE 112	Benin	4*x*
TDa 3901	Biowonkourou	Benin	4*x*
TDa 3701	Mpalakala	Congo	4*x*
TDa 3703	Unkown	Congo	4*x*
TDa 1239	IC 28	Côte d'Ivoire	4*x*
TDa 1194	IC 20	Côte d'Ivoire	4*x*
TDa 3925	N'zasegula	Côte d'Ivoire	8*x*
TDa 1250	IC 3	Côte d'Ivoire	8*x*
TDa 3128	EQ-89—23	Equatorial Guinea	4*x*
TDa 1313	PS/89/168	Gabon	8*x*
TDa 3275	Mensmfi	Ghana	4*x*
TDa 1333	Akwa	Ghana	4*x*
TDa 3269	Alamunkpiti	Ghana	4*x*
TDa 2849	Kronkosi	Ghana	4*x*
TDa 2846	Adiamawoba	Ghana	4*x*
TDa 1297	Dansiesumne 1	Ghana	4*x*
TDa 2844	Dansiesumne 2	Ghana	4*x*
TDa 2851	Akwa 2	Ghana	4*x*
TDa 3914	Souwanrou	Nigeria	4*x*
TDa 1286	Fayinka	Nigeria	4*x*
TDa 1429	UYT TDA 96	Nigeria	4*x*
TDa 1431	86/00611	Nigeria	4*x*
TDa 1347	85/0253	Nigeria	4*x*
TDa 1404	(29) 40	Nigeria	4*x*
TDa 1391	UM 680	Nigeria	4*x*
TDa 1442	HT 86/88682	Nigeria	4*x*
TDa 4041	Weredede	Nigeria	4*x*
TDa 1416	TDA. D3 ALATA	Nigeria	4*x*
TDa 1467	86/00611	Nigeria	4*x*
TDa 1454	87/0305—19	Nigeria	4*x*
TDa 3743	BN 301	Nigeria	4*x*
TDa 1430	PYT 85/0236	Nigeria	6*x*
TDa 1441	UYT TDA 96	Nigeria	8*x*
TDa 3920	Aluwinrin	Nigeria	8*x*
TDa 4142	Ngaobule	Sierra Leone	4*x*
TDa 4127	Unkown	Sierra Leone	4*x*
TDa 4134	Unkown	Sierra Leone	4*x*
TDa 4146	Yamssiegbolie	Sierra Leone	4*x*
TDa 4139	Yamsigboi	Sierra Leone	4*x*
TDa 3202	Kaki AB-6 972	Togo	4*x*
TDa 1096	Kaki A 86	Togo	4*x*
TDa 3234	Adigo 303	Togo	4*x*
TDa 1165	Unkown	Togo	4*x*
TDa 1201	Coulou	Togo	4*x*
TDa 3231	Gnagnassi40	Togo	4*x*
TDa 1066	Fanamawe T25	Togo	4*x*
TDa 1069	Gnalabi	Togo	4*x*
TDa 1210	Kpent 112	Togo	4*x*
TDa 1152	Sova2B 50	Togo	4*x*
TDa 1295	TcussenKN 19	Togo	4*x*
TDa 3187	Tfigou D-9—1156	Togo	4*x*
TDa 1060	Tonfou	Togo	4*x*
TDa 3168	Tsrorpa154	Togo	4*x*
TDa 2869	Guete 43	Togo	4*x*
TDa 1123	Tabere T-39	Togo	4*x*
TDa 1209	Tsrokpa784	Togo	4*x*
TDa 1065	Unkown	Togo	4*x*
TDa 1103	Fabangasot 135	Togo	4*x*
TDa 1159	Awe 71	Togo	4*x*
TDa 1021	Betebete	Togo	4*x*
TDa 1108	Lambor mande KN 82	Togo	4*x*
TDa 1164	Fetiou BH 34	Togo	4*x*
TDa 3215	Ogbo 186	Togo	4*x*
TDa 3163	Kabanga S-1 639	Togo	8*x*
TDa 1032	Gnagnassion	Togo	8*x*
TDa1124	Kere 2B 65	Togo	8*x*
TDa 3221	Afassetchissem KN-91 1321	Togo	8*x*
TDa 1237	226	Togo	8*x*

the negative effect of cytosolic compounds present in the leaves of *D. alata* and thus, improve the overall quality of the fluorescence histograms (Loureiro et al. 2006). One millilitre of nuclear suspension was recovered and filtered through an 80-µm nylon filter to remove cell fragments and large debris. Nuclei were stained with 50 µg ml^{-1} propidium iodide (PI,

Fluka, Buchs, Switzerland), and 50 µg ml^{-1} RNase (Fluka) was added to the nuclear suspension to prevent staining of double-stranded RNA. Samples were incubated on ice and analysed within 10 min of preparation. *Solanum lycopersicum* L. (2C = 1.96 pg DNA; Doležel et al. 1992) was chosen as reference standard. According with previous estimations of nuclear DNA content in yams (2C = 0.88–2.88 pg; Hamon et al. 1992), the chosen reference standard presents a close but non-overlapping genome size, ideal for the ploidy level inference in *D. alata*.

Flow cytometric analyses

Nuclear samples were analysed using a Coulter EPICS XL flow cytometer (Beckman Coulter®, Hialeah, FL, USA). The instrument was equipped with an air cooled argon-ion laser tuned at 15 mW and operating at 488 nm. PI fluorescence was collected through a 645-nm dichroic long-pass filter and a 620-nm band-pass filter. Prior to analysis the instrument was checked for linearity with Flow-Check fluorospheres (Beckman Coulter®). The amplifier system was adjusted so that the G_0/G_1 peak of nuclei isolated from tetraploid individuals was set at channel 200 in a 1,024 channels scale. The instruments settings, i.e., voltage and gain, were kept constant throughout the experiment. The results were acquired using the SYSTEM II software (version 3.0, Beckman Coulter®).

In most cases, at least 3,000 nuclei were analysed per sample. The ploidy level was estimated by comparing the mean fluorescence intensity of nuclei of sample material with that of the reference standard. In the majority of samples, an external standardization procedure was followed. When doubts on the ploidy level assignment occurred (deviations of more than 10.0% from the expected mean fluorescence), a new sample with co-chopped plant material and reference standard was prepared. Also, in some cases, samples of unknown ploidy were prepared together with control individuals of known ploidy level.

Results and discussion

Clearly defined histograms for accurate determination of ploidy levels were obtained following flow cytometric analysis of intact leaf nuclei (Fig. 1). Table 1 shows the ploidy level inference for each accession analysed in this investigation. The results inferred that 63 of the accessions are tetraploid, one is hexaploid and 10 are octoploid (Table 2). The mean nuclear DNA content for accessions of *D. alata* in which individual plants were analysed simultaneously with *S. lycopersicum* (as an internal reference standard) was of 1.18 ± 0.02 pg/2C, which according with the available literature (2C = 4*x* = 1.15 pg, Arumuganathan and Earle 1991) corresponds to the tetraploid level. For other plants, in which tetraploid accessions were used as a reference, channel number values for tetraploid, hexaploid and octoploid accessions approximated 1:1.5:2 ratios, giving no doubts on the ploidy level inferences that were made (Fig. 1). The coefficients of variation (CV) values of the G_0/G_1 peaks were usually within the limit of 5% (Table 2). With exception of tetraploid accessions, where a higher heterogeneity was obtained, CV values were below 3.5%.

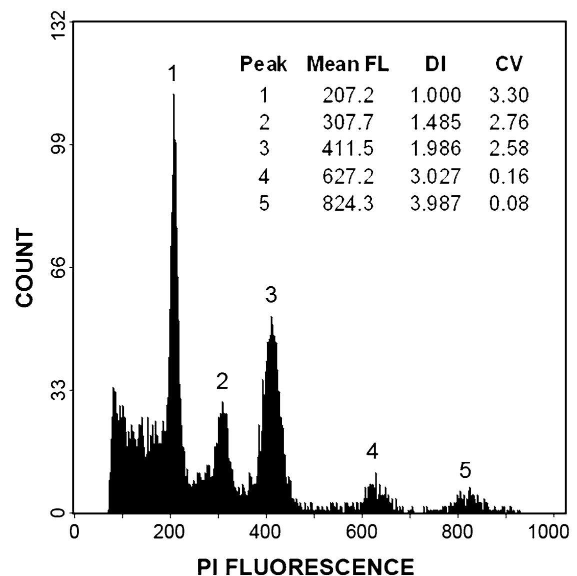

Fig. 1 Histogram of relative fluorescence intensity of nuclei isolated simultaneously from three accessions of *Dioscorea alata* L. with different ploidy levels: tetraploidy (peaks 1 and 3), hexaploidy (peaks 2 and 4) and octoploidy (peaks 3 and 5). Peaks 1, 2 and 3 correspond to nuclei at the G_0/G_1 phase, while peaks 3, 4 and 5 to nuclei at the G_2 phase. The mean channel number (Mean FL), DNA index (DI = mean channel number of sample/mean channel number of tetraploid accession), and coefficient of variation (CV, %) value of each peak are also given

Table 2 Ploidy level analysis in *Dioscorea alata* L.

Ploidy level	No. of individuals	DI	CV (%)	CV range (%)	
				Min.	Max.
Tetraploidy	63 (85.1%)	0.59	3.76	2.47	6.51
Hexaploidy	1 (1.4%)	0.88	2.95	–	–
Octoploidy	10 (13.5%)	1.18	2.04	2.40	3.15
Total	74	–	3.60	2.40	6.51

The DNA index (DI = mean channel number of sample/mean channel number of reference standard) and mean and range of the coefficient of variation CV (%) obtained are also given

Earlier studies highlighted the occurrence of different levels of polyploidy in this species (Sharma and De 1956; Martin and Ortiz 1966; Baquar 1980; Hamon et al. 1992; Gamiette et al. 1999; Egesi et al. 2002). In comparison with those studies, our results do not affirm the earlier cytogenetic observations of Sharma and De (1956) and Hamon et al. (1992), where individuals with a continuous range of ploidy levels ($2n = 3x$, $3.5x$, $4.5x$, $7x$, $8.5x$) were reported. However, they are consistent with the results of the study of Gamiette et al. (1999) who also reported the occurrence of tetraploid, hexaploid and octoploid individuals in their test samples, with tetraploids being the most commonly found cytotype. In another large-scale screening of *D. alata* germplasm, Egesi et al. (2002) showed that the majority of plants were hexaploid, with a smaller percentage of tetraploids, and no octoploids. No mixoploid individuals were identified in the composite set, which together with the similar observations of Gamiette et al. (1999) and Egesi et al. (2002) may suggest a genetic stability over time that offers breeding advantages.

Essad (1984) reported a base chromosome number of $x = 10$ in all the Asian species of *Dioscorea*. This base chromosome number was only found in 52% of the African species and in 13% of the American species. Recent data using microsatellite markers revealed a basic chromosome number of $x = 20$ in African yam *Dioscorea rotundata* Poir. (Scarcelli et al. 2005) and in American yam *Dioscorea trifida* L.f. (Bousalem et al. 2006). These new discoveries rouse doubts on the validity of the current status of ploidy data in the *Dioscorea* genus. However, as these results were obtained in African and American species, and as there is still some paucity on the data of Asiatic species, like *D. alata*, the findings of Essad (1984) remain, up to this moment, valid. Future phylogenetic works should concentrate on Asian species, in order to elucidate this issue.

The high percentage of tetraploids (85.1%) observed in this composite set is in agreement with the results of Gamiette et al. (1999), which together with an absence of odd ploidy levels ($3x$, $5x$ and $7x$) suggest that polyploidization by fusion of reduced (n) and unreduced ($2n$) gametes may be a rare phenomenon in *D. alata*. Whether it seems probable that octoploid individuals originated through autopolyploidy and that hexaploid ones resulted from the crossing of tetraploid and octoploid cultivars, the origin of the tetraploids is still not clear. As previously described for other clones, no diploid individuals were detected within our set of plants, which may suggest an allopolyploid origin of *D. alata* tetraploids. Barrau (1965) suggested that *D. alata* derived from two ancestral species close to *Dioscorea hamiltonii* Hook.f. and to *Dioscorea persimilis* Prain et Burkill.

As it was already shown in Egesi et al. (2002), no association between ploidy level and place of cultivation of the test materials was detected (Table 1). Until this moment, the highest ploidy level already observed in *D. alata* was octoploidy ($2n = 8x = 80$), which may suggest that the maximum ploidy level that this species can tolerate may have been reached. However, if so, the mechanism(s) that underlie this limit for multiplying the nuclear genome is to us unknown.

In conclusion, the data presented in this paper are noteworthy, as flow cytometry has been successfully used to determine ploidy level of the composite collection of *D. alata*. It represents an essential knowledge for the effective breeding and conservation of the species, as well as, it provides new insights on the phylogeny and evolution of *D. alata*. Furthermore, our findings set the foundation for the needed sustainability in long-term breeding strategies for this valuable composite set of *D. alata.*

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