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ORIGINAL PAPER

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Segregation patterns of isozyme loci and microsatellite markers show the diploidy of African yam *Dioscorea rotundata* (2*n* = 40)

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Abstract The cultivated yam species *Dioscorea rotundata* ($2n = 40$) has been considered by most authors as a tetraploid species with a basic chromosome number of ten. In this paper, we analysed the segregation of two isozyme loci and six microsatellite markers in the progeny of a self-fertilised monoecious plant. For the eight markers, segregation patterns could be explained by only two genetic models: diploidy or tetraploidy with two null alleles. Given the nature of studied markers, the most parsimonious hypothesis was that the parental plant was diploid. These results, data from a diversity survey and results of other authors led to the conclusion that *D. rotundata* is a diploid species.

Keywords Yam · *Dioscorea rotundata* · Ploidy level · Microsatellite · Isozyme · Segregation

Introduction

Yam (*Dioscorea* sp.) is cultivated for its tuber production in tropical countries. The most cultivated species in Africa, *Dioscorea rotundata* (section *Enantiophyllum*), has 40 chromosomes. Based on indirect conclusions, such as the existence of $2n = 20$ yam species in the section *Stenophora* (Mignouna et al. 2002), the tetraploidy of this species has been assumed by most authors (e.g. Dansi et al. 2000; Gamiette et al. 1999; Hamon et al. 1992; Miege 1952; Mignouna et al. 2002; Zoundjihekpon et al. 1990).

Under this assumption, one would therefore anticipate the detection in individual genotypes of up to four alleles per DNA marker locus in diversity studies of *D. rotundata*, as observed in several tetraploid species (e.g. *Borderea* sp. and *Medicago* sp. by Segarra-Moragues et al. 2004 and Diwan et al. 2000, respectively). However, when doing a preliminary survey of *D. rotundata* diversity with 11 microsatellite loci, we found only one or two alleles per locus in every genotype. Although this does not rule out the possibility of this species being a tetraploid, it strongly suggests considering a diploid hypothesis.

Daïnou et al. (2002) were the first authors to propose diploidy of *D. rotundata*. They mostly based their conclusion upon the segregation ratios observed for isozymes in the progeny of a self-fertilised monoecious *D. rotundata* plant. Diploid segregations of AFLP and isozymic markers were also observed in progenies of *D. rotundata* plants (Mignouna et al. 2002; Zoundjihekpon et al. 1994), although these authors did not use their results to challenge the tetraploid status of *D. rotundata*.

However, the above papers do not permit a conclusion as to the diploidy of *D. rotundata*. The hypothesis of null alleles in tetraploid genotypes needs to be considered as a possible explanation to the observed segregations. It was considered, but not tested, by Daïnou et al. (2002).

The present work aimed at reaching reliable conclusions on the ploidy level of *D. rotundata*, which is a basic biological data for yam breeders. Moreover, the increasing use of microsatellite markers for the diversity analysis of African yam species makes it necessary to get a better knowledge of their genetic determinism. In this study, we analysed the segregation patterns of six microsatellite loci in the selfed progeny of a heterozygote monoecious plant showing two alleles at each of these. We also re-analysed the segregation patterns of the two isozyme markers obtained by Daïnou et al. (2002) on the same progeny. All potential segregation ratios expected

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in the self-fertilisation of a heterozygote genotype under diploid, allo- and autotetraploid models were tested. We first showed that only the diploid model and the allotetraploid model with two null alleles were consistent with the observed ratios. We then explained why, given the nature of studied markers and data of a diversity survey, we came to the conclusion that *D. rotundata* ($2n = 40$) is a diploid species.

Material and methods

Segregation analysis

Plant material

Although *D. rotundata* is a dioecious species, some monoecious plants were found in the variety Gnidou. These plants allowed us to produce a selfed progeny.

Yam is a vegetatively propagated crop. Farmers define yam varieties with morphological criteria. A given variety may then encompass several clones, even at the field level. To ensure the production of a selfed progeny despite the potential polyclonality of Gnidou variety, several clonal seedlings were first produced by division of a single tuber of a *D. rotundata* plant. This plant was monoecious with $2n = 40$ chromosomes (Dansi et al. 2000). The clonal plants were grown in an isolated field. The seeds were collected and were assumed to result from self-fertilisation or from cross-pollination between plants, which was equivalent to a self-fertilisation. Reproductive isolation of the field was confirmed by the absence in the progeny of any other allele than the parental ones.

Isozymes

Isozyme analysis was performed in the Laboratory of Genetics in Cotonou. Seeds from the Gnidou progeny were germinated. Extraction of proteins was done using 1-month-old leaves crushed in the buffer slightly modified from Hamon and Touré (1982) (no potassium cyanide and only 1% of mercaptoethanol). Individuals analysed for isozyme segregations were not the same as those used for microsatellite studies.

Two isozymes were analysed: 6-phosphogluconate dehydrogenase [(PGD) EC 1.1.1.44] and phosphoglucomutase [(PGM) EC 5.4.2.2]. Fifty-five and 208 other individuals were analysed for PGM and PGD, respectively. A 13% starch gel was prepared using a solution of histidine (5 m *M*) and NaCl (2.5 m *M*) at pH 6.25. Electrophoresis was conducted using a buffer of citric acid (0.41 m *M*) at pH 6 during 5 h at 100 mA. Enzyme assays were conducted as described by Second and Trouslot (1980).

Microsatellite markers

Ninety individuals, corresponding to 90 seeds of the progeny, were analysed at IRD Montpellier for segregation at microsatellite loci. DNA extraction was done from single seeds. Seeds were broken and treated three times with sorbitol buffer [0.35 *M* sorbitol, 0.1 *M* Tris-HCl, 5 m *M* ethylenediaminetetraacetic acid (EDTA) and 0.5% bisulfite for the first treatment only with centrifugation at 9,000 *g*, 10 min, 4°C]. Samples were incubated in Matab buffer (1.2 *M* NaCl, 0.1 *M* Tris-HCl, 20 m *M* EDTA and 4% Matab) for 4 h at 65°C. Samples were treated twice with chloroform/isoamyl 24:1 (centrifugation 9,000 *g*, 10 min, 10°C). DNA was precipitated with 0.6 vol of isopropanol (centrifugation 14,000 *g*, 15 min, 4°C), washed in 70% ethanol, dried and re-suspended in sterile water.

Twenty microsatellite primers, 17 defined in our laboratory (unpublished data) and three defined by Mignouna et al. (2003), were amplified in the Gnidou parent. Two alleles at most were observed at each locus. Fourteen loci were monoallelic. The progeny was analysed for the six microsatellite loci showing two alleles in the parental genotype (loci 2D08, 3D06, 3F04, 3F10, 3F12 and YM13). PCRs were done in 10 µl with dNTP (200 µ*M*), *Taq* polymerase (0.05 U) in its buffer (1X), $MgCl_2$ (2.5 m *M*), forward primer tailed (3F04-M13, 2D08-T7; 0.01 µ*M*) or labelled (3D06, 3F10, 3F12, YM13; 0.25 µ*M*), reverse primer (0.15 µ*M* for 3F04 and 2D08, 0.25 µ*M* for the other) and M13 or T7 labelled primer (0.15 µ*M*) for 3F04 and 2D08. Amplifications were performed by 35 PCR cycles (94°C for 3 min, 94°C for 1 min, 56°C for 1 min, −1°C per cycle, 72°C for 1 min for five cycles, 94°C for 1 min, 51°C for 1 min, 72°C for 1 min for 30 cycles and 72°C for 8 min). The migration was done using an ABI Prism 3100 (Applied Biosystems, Foster City, Calif., USA). The analysis of intensity profiles to score the alleles was done with software Genescan and Genotyper (Applied Biosystems).

Models for statistical analysis

Let A and B be the two co-dominant alleles at one locus. If the phenotype of the parent is (AB), then the parental genotype could be AB if the species is diploid, or AAAB, AABB and ABBB if the species is tetraploid. Three phenotypes are expected in the progeny of self-fertilisation: (A) corresponding to genotypes AA and AAAA; (AB) corresponding to genotypes AB, ABBB, AABB, and AAAB; and (B) corresponding to genotypes BB and BBBB. If O is a null allele, potential parental genotypes are AABO, ABBO and ABOO. Ten new genotypes are then expected in progeny: AAAO, AAOO, AOOO, BBBO, BBOO, BOOO, AABO, ABBO, ABOO and OOOO. The expected proportions of each phenotype in the progeny under diploid and tetraploid model with disomic (allotetraploid model) and tetrasomic (autotetraploid model) inheritance are given for each possible parental genotype in Table 1.

Goodness of fit of observed frequencies to expected proportions in each of these models were tested using a Khi2 test or a Fisher's exact test if one expected frequency was less than 5.

Table 1 Expected ratios of phenotypes in the progeny of a self-fertilisation for all potential parental genotypes with a phenotype (AB)

Genetic model for parental genotype[a]	Phenotypic ratio expected in progeny				
		(OO)	(AA)	(AB)	(BB)
AB	1:2:1	0	1/4	1/2	1/4
AA/BB	1	0	0	1	0
AB/AB	1:14:1	0	1/16	14/16	1/16
AA/AB, AAAB	1:3	0	1/4	3/4	0
AB/BB, ABBB	3:1	0	0	3/4	1/3
AO/AB	4:11:1	0	4/16	11/16	1/16
OB/AB	1:11:4	0	1/16	11/16	4/16
AA/OB	1:3	0	1/4	3/4	0
AO/BB	3:1	0	0	3/4	1/4
AO/BO	1:3:9:3	1/16	3/16	9/16	3/16
AB/OO	1:2:1	0	1/4	1/2	1/4
AABB	1:34:1	0	1/36	34/36	1/36
AABO	9:26:1	0	9/36	26/36	1/36
ABBO	1:26:9	0	1/36	26/36	9/36
ABOO	1:8:19:8	1/36	8/36	19/36	8/36

[a]In case of allotetraploidy, the two genomes segregate independently (disomic segregation). This was represented by separating the two genomes *with a forward slash*. For example, AA/BB means that the genotype is AA on the first genome and BB on the second one. In case of autotetraploidy, the two genomes segregate together (tetrasomic segregation) and were represented *without a forward slash*, for example, AABB

Independence of microsatellite loci was tested using Mapdisto 1.3.2 (available at http://mapdisto.free.fr/).

Diversity survey

One hundred and eighty-one samples of *D. rotundata*, representing the most cultivated varieties of Benin (Dansi et al. 2000), 245 *D. abyssinica* and 63 *D. praehensilis* were collected in Benin. *D. abyssinica* and *D. praehensilis* were considered by Hamon et al. (1997) and Terauchi et al. (1992) as wild relatives of *D. rotundata*. These two species have been assumed to be tetraploid, with $2n = 40$ chromosomes (Miege 1952).

Eleven microsatellites (1A01, 3B12, 1C12, 2C05, 2D06, 2D08, 2E07, 2E09, 1F08, 3F04 and 3G04, unpublished data) were amplified as explained above. In this paper, only data on heterozygosity and number of alleles per locus are presented.

Results

Segregation analysis

Isozyme loci

The PGM banding pattern of the parent showed four bands, two slow and two fast (Fig. 1a). The two slow bands showed no polymorphism in the progeny. The segregation of the two fast bands (1/2) showed three phenotypes, (1), (1/2) and (2), in the proportions given in Table 2. The Khi2 tests rejected all models except those of diploidy and tetraploidy with two null alleles.

The PGD banding pattern of the parent showed three bands (1/2/3, Fig. 1b). In the progeny, we observed one phenotype with the fast band (1), one with the three parental bands (1/2/3) and one with two bands (1/3, Fig. 1b) in proportions in Table 2. This banding pattern was previously observed by Zoundjihekpon et al. (1994).

PGD was described by Weenden and Wendel (1989) as dimeric and coded by two genes. We could explain the banding patterns when considering that: (1) the parent has two alleles for the first gene, coding for two monomeres which bind to produce two homodimeres (corresponding to the bands 1 and 3) and one heterodimere (corresponding to the band 2), and (2) the parent is homozygote for the second gene which produces a homodimere that co-migrates with the homodimere corresponding to the band 1 of the former gene. Under these assumptions, the Khi2 tests rejected all models except the diploid model and the tetraploid models with two null alleles, ABOO and AB/OO (Table 3).

Microsatellite loci

Microsatellite profiles of the parent showed only two alleles for each of the six non-monoallelic loci. The observed phenotypes were: (138/144) (bp) for 3F04, (353/371) for 2D08, (143/145) for 3D06, (156/158) for 3F10, (162/164) for 3F12 and (220/227) for YM13 (Fig. 2a). In the progeny, three phenotypes were obtained for each

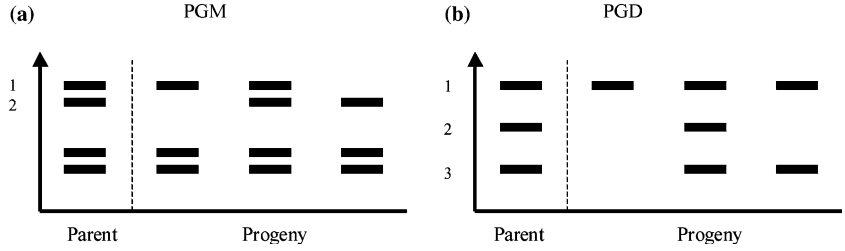

Fig. 1 Banding patterns obtained in the Gnidou parent and in the progeny for the isozymic loci phosphoglucomutase (*PGM*) and 6-phosphogluconate dehydrogenase (*PGD*). Numbers are those of the bands, as used in the text

Table 2 Phenotypes observed in the parent and in the progeny, and observed frequencies

		Phenotype of parent	Phenotype of progeny and observed frequencies		
Microsatellite loci[a]	2D08	(353/371)	19 (353)	44 (353/371)	26 (371)
	3D06	(143/145)	25 (143)	41 (143/145)	24 (145)
	3F04	(138/144)	18 (138)	48 (138/144)	18 (144)
	3F10	(156/158)	19 (156)	45 (156/158)	26 (158)
	3F12	(162/164)	26 (162)	43 (162/164)	21 (164)
	YM13	(220/227)	24 (220)	40 (220/227)	26 (227)
	Global analysis	(AB)	107(AA)	213(AB)	129(BB)
Isozyme loci	PGD	(1/2/3)	51 (1)	104 (1/2/3)	53 (1/3)
	PGM	(1/2)	11 (1)	26 (1/2)	18 (2)

[a]For microsatellite loci, phenotypes are given in base pairs. Frequencies used for the global analysis were the sum of frequencies of phenotypes observed at loci 2D08, 3D06, 3F10, 3F12 and YM13

Table 3 *P*-values obtained for a Khi2 test to compare the expected ratios given in Table 1 and the observed frequencies given in Table 2

Genetic model for parental genotype	Expected ratio	*df*	*P*-values for Khi2 tests of goodness of fit								
			Microsatellite loci							Isozyme loci	
			2D08	3D06	3F04	3F10	3F12	YM13	Global analysis	PGD[a]	PGM[b]
AB	1:2:1	2	**0.424**	**0.573**	**0.693**	**0.580**	**0.693**	**0.549**	**0.189**	**0.981**	**0.378**
AA/BB	1	–	–	–	–	–	–	–	–	–	–
AB/AB	1:14:1	2	0	0	0	0	0	0	0	0	0
AA/AB, AAAB	1:3	–	–	–	–	–	–	–	–	–	–
AB/BB, ABBB	3:1	–	–	–	–	–	–	–	–	–	–
AO/AB	4:11:1	2	0	0	0	0	0	0	0	0	0.002
OB/AB	1:11:4	2	0	0	0	0	0	0	0	0	0.036
AA/OB	1:3	–	–	–	–	–	–	–	–	–	–
AO/BB	3:1	–	–	–	–	–	–	–	–	–	–
AO/BO	1:3:9:3	3	**0.116**	0.008	0.013	0.008	0.040	0.005	0	0.000	**0.122**
AB/OO	1:2:1	2	**0.424**	**0.573**	**0.693**	**0.580**	**0.693**	**0.549**	**0.189**	**0.981**	**0.378**
AABB	1:34:1	2	0	0	0	0	0	0	0	0	0
AABO	9:26:1	2	0	0	0.001	0	0	0	0	0	0
ABBO	1:26:9	2	0	0	0.001	0	0	0	0	0	0.004
ABOO	1:8:19:8	3	**0.397**	**0.317**	**0.582**	**0.404**	**0.364**	**0.267**	0	**0.056**	**0.492**

If the expected frequencies were less than 5, a Fisher's exact test was done. Non-significant *P*-values ($P > 0.050$) are in *boldface*. Models were rejected without test (–) when the expected proportion for one observed phenotype was null

[a]*PGD* 6-Phosphogluconate dehydrogenase

[b]*PGM* Phosphoglucomutase

locus, corresponding to two different homozygotes and one heterozygote. The observed frequencies of each phenotype are given in Table 2. As for isozymic loci, only the diploid model and the tetraploid models with two null alleles were compatible with the observed segregations.

All microsatellite loci were found independent using a LOD score equal to 4, but 3F04 and 3F12 were loosely linked using a LOD score equal to 3 ($r = 28.6$ cm, Kosambi distance).

We used this result to make a global analysis by pooling segregations of loci 2D08, 3D06, 3F10, 3F12 and YM13. Global multilocus frequencies for phenotypes (AA) and (BB) were computed to get the most unbalanced totals, by summing for (AA) the smallest frequencies of homozygotes at each locus, and for (BB) the highest frequencies of homozygotes for each locus (Table 2). From this multilocus analysis, only two models were accepted (Table 3): diploidy, and allotetraploidy with null alleles (AB/OO). These two models could not be distinguished since expected ratios in the progeny were the same (1:2:1) for both models.

Diversity survey

Microsatellites loci revealed a high diversity. A total of 250 alleles were amplified in the three species in 11 loci (Table 4). The mean number of alleles varied from 11.5 for *D. rotundata* to 19.4 for *D. abyssinica* and all loci had four or more alleles in each species. Results supported the diploidy hypothesis. Almost all individuals displayed only one or two alleles per locus. In heterozygote individuals, peak areas were similar and compatible with the presence of one copy of each allele (Fig. 2b). Out of the 5,172 data points produced, only eight (0.15%) could be interpreted as three or four alleles and were found in six individuals out of the 489 studied (one *D. rotundata*, two *D. abyssinica* and three *D. praehensilis*) at only one or two loci for each of these individuals (loci 1A01, 2C05, 2E07, 2E09 and 1F08). Presence of supernumerary alleles was previously observed in diploid species like maize and grapevine (e.g. Franks et al. 2004; Matsuoka et al. 2002) and could be explained by DNA contamination, amplification of a non-specific allele or chimerism.

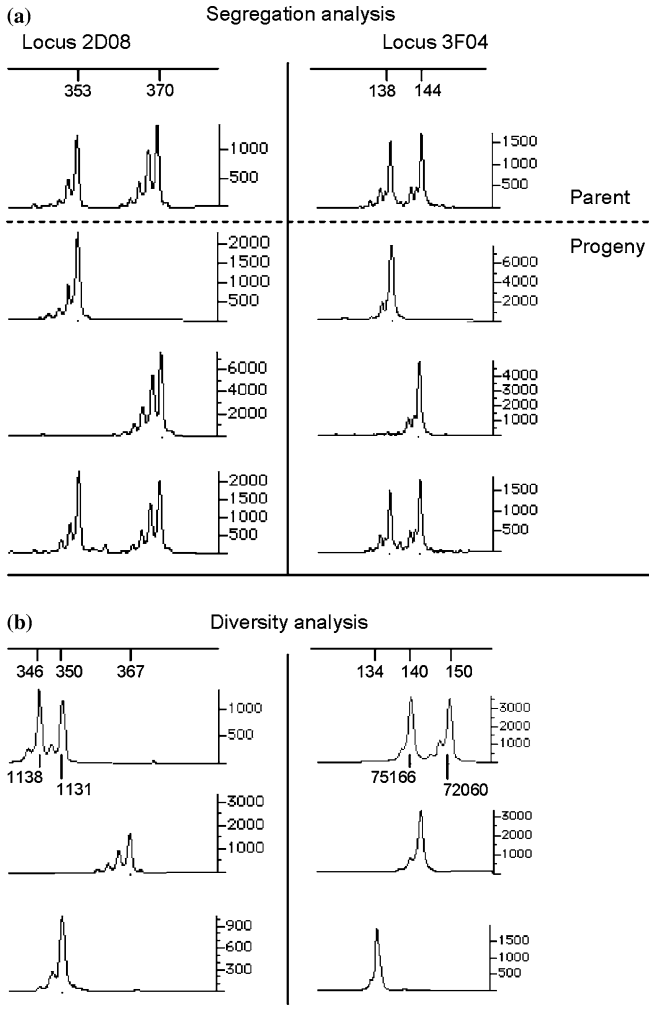

Fig. 2 Examples of electropherogram obtained for microsatellite loci 2D08 and 3F04 with the automated sequencer ABI Prism 3100 and analysed with software Genotyper. **a** Banding patterns obtained in the segregation analysis. The parent and one example of each genotype obtained in progeny are represented. **b** Example of genotypes obtained in the diversity analysis. For heterozygote individuals, *annotation* indicates the peak area

Discussion

Eight markers were analysed in the selfed progeny of the Gnidou parent, two isozyme and six microsatellite loci. If we consider all the tests done, only two genetic models were consistent with observed segregations: the diploid model and the allotetraploid model with null alleles on one of the two genomes (AB/OO).

The allotetraploid model is unlikely for several reasons. Null alleles are common for isozyme (Weenden and Wendel 1989) and microsatellite loci (Dakin and Avise 2004 for a review), but it would be unlikely to observe in one genome eight randomly sampled markers homozygote for null alleles (this also applies to the autotetraploid model with two null alleles we rejected after the multilocus analysis).

Only a complex set of hypotheses could explain why segregations at both isozyme and microsatellite loci reflect the allotetraploid model with null alleles. For isozyme markers, gene silencing can produce a diploid pattern (Wendel 2000 for a review). This cannot be excluded from our data, but it should then be associated with the non-detection of microsatellite polymorphism in one of the two genomes. As microsatellites are generally specific to a small number of species, we might have amplified only one genome. However, the microsatellites we used were initially chosen to amplify in other very different yam species (unpublished data): *D. alata* and *D. nummularia* (Asian species), *D. trifida* (Caribbean species), *D. bulbifera*, *D. abyssinica* and *D. praehensilis* (African species). If there were a non-amplified genome in *D. rotundata*, we would have to conclude that this genome is more distant to African species than Asian or Caribbean species are. It sounds very unlikely, even though the origin of *D. rotundata* is not clearly assessed (Hamon et al. 1997; Terauchi et al. 1992).

Overall, as suggested by the preliminary analyses of Dainou et al. (2002), the diploidy of *D. rotundata* is the most parsimonious hypothesis.

Table 4 Allele diversity at 11 microsatellite loci in the cultivated species *D. rotundata* and the two wild species *Dioscorea abyssinica* and *D. praehensilis*. H_{obs} Observed heterozygosity

Loci	*D. rotundata*		*D. abyssinica*		*D. praehensilis*		Total no. of alleles
	H_{obs}	No. of alleles	H_{obs}	No. of alleles	H_{obs}	No. of alleles	
1A01	0.16	6	0.62	14	0.75	12	17
3B12	0.29	9	0.26	12	0.43	6	15
1C12	0.63	8	0.11	8	0.22	4	11
2C05	0.81	14	0.80	34	0.82	23	36
2D06	0.84	13	0.83	19	0.73	19	22
2D08	0.84	14	0.84	26	0.73	15	28
2E07	0.83	18	0.78	27	0.68	25	33
2E09	0.17	8	0.80	17	0.70	11	19
1F08	0.61	8	0.65	13	0.76	9	14
3F04	0.69	14	0.82	24	0.85	20	29
3G04	0.52	15	0.54	19	0.56	17	26
Total	–	127	–	213	–	161	250
Mean	0.58	11.5	0.64	19.4	0.66	14.6	22.7

Is this conclusion weakened by the fact that it was drawn from the study of the progeny of a monoecious *D. rotundata* plant, which is an exception in this normally dioecious species? In other words, could diploidy and monoecy of the parental plant be related? As far as we know, literature does not provide any example to support this hypothesis. Exceptional bisexual plants were found in other dioecious species (Rottenberg 2000). Investigations on the interactions between ploidy and sexual systems have led to heterogeneous conclusions (Miller and Venable 2000; Pannel et al. 2004), but were conducted in species complexes that include species with different chromosomes numbers. The case of Gnidou genotype is different as it displays the same chromosome number as dioecious *D. rotundata* plants. We thus think that the conclusion on its diploidy can be extended to the *D. rotundata* genotypes with the same chromosome number ($2n = 40$).

This is supported by the consistency with the diploid model of results that were obtained by other authors from studies of dioecious *D. rotundata* plants. Zoundjihekpon et al. (1994) observed in progenies of controlled hybridisations of dioecious *D. rotundata* a diploid segregation for isocitrate dehydrogenase, esterase, saccharopine dehydrogenase and malate dehydrogenase enzyme systems.

In the same species, Mignouna et al. (2002) analysed the segregation of 341 AFLP markers to develop a genetic linkage map. Citing Hahn (1995), they concluded that his allotetraploidy hypothesis was supported by the disomic inheritance obtained in the studied progenies and because chromosome pairing showed 20 bivalents during meiosis. It must first be noted that Hahn's evolutionary scenario proposes the autotetraploidy of *D. rotundata*, not its allotetraploidy. More crucial is the fact that the allotetraploid model cannot be accepted from these AFLP data. As mentioned by these authors themselves, Mignouna et al. (2002) obtained diploid segregations (i.e. not only disomic segregations) and they eventually considered *D. rotundata* as diploid for the mapping analysis.

Finally, we showed that the diploidy of *D. rotundata* was also supported by the allele diversity analysis. The survey of a large number of plants with 11 highly polymorphic microsatellite loci showed that plants of cultivated species *D. rotundata* and wild species *D. abyssinica* and *D. praehensilis* displayed at most two alleles at each locus. As discussed above, the hypothesis of non-amplification of a genome is very unlikely given the way microsatellite primers were constructed and selected.

Conclusion

D. rotundata ($2n = 40$) can be considered as a diploid species with a basic chromosome number $x = 20$. This is strongly supported by the sum of observations obtained in our study and in those of authors already cited: (1) only one or two alleles per genotype were observed at microsatellite loci in diversity surveys; (2) isozyme patterns could be explained by a diploid model; (3) only bivalents were observed during meiosis; (4) diploid segregations were obtained in isozymic, AFLP and microsatellite markers; and (5) the nature of genetic markers used in our study makes the allotetraploid model with null alleles extremely unlikely.

The extant diploidy of *D. rotundata* does not preclude its allotetraploid origin. Diploidisation of genomes after their polyploidisation has been documented for several wild and cultivated plant genomes. For example, *Brassica* species, soybean and maize appear to be highly diploidised paleopolyploids (see Wendel 2000 and Gaut et al. 2000 for a review). Such events could explain the co-existence within the genus *Dioscorea* of the diploid 2 $n = 40$ *D. rotundata* together with diploid 2 $n = 20$ species such as *D. tokoro* and *D. gracillima* (section *Stenophora*). Our diversity surveys suggest that the wild species *D. abyssinica* and *D. praehensilis* could also be diploid. Research is needed to re-examine the evolutionary scenario of African yams.

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