



Plant Sex Prediction Using Genetic Markers in Cultivated Yams (*Dioscorea rotundata* Poir.) in Benin

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Abstract: *Dioscorea rotundata* Poir. is mainly dioecious but it also produces monoecious individuals. Recently two genetic markers were proposed to determine the sex in this species. We tested them in 119 individuals from 101 different cultivars of the national collection of Benin to verify whether they can predict the sex observed in the field. Among the analyzed individuals, 72 were male, 37 were female, 7 were monoecious and 3 were non-flowering. Our results showed that the marker sp16, associated with the W allele (female allele), was present in all female individuals but also in more than 42% of male individuals. Thus, while the absence of sp16 confirmed the male sex of the individuals, its presence did not allow sex discrimination. The marker sp1 allowed the identification of four genotypes (AA, AB, BB and AC) in the analyzed individuals with AA and AB being the most represented. Although AA was observed in 62.16% of female individuals and AB in 83.33% of male individuals, we did not observe a clear correlation between sp1 genotypes and sex identity. We concluded that the tested markers did not allow a clear sex discrimination in Beninese *Dioscorea rotundata* cultivars. Our results also suggest that Beninese *D. rotundata* cultivars have adopted a male XX/XY heterogametic system that is undergoing reorganization.

Keywords: dioecy; monoecy; sex determination; sexual chromosome; yam

1. Introduction

The species *Dioscorea rotundata* Poir. is one of 600 species of the genus *Dioscorea* in the family Dioscoreaceae [1]. This genus is commonly known as yam and *D. rotundata* is described as white Guinea yam [2,3]. This species is also named *Dioscorea cayenensis* subsp. *rotundata* Poir. J. Miège or *Dioscorea cayenensis*—*D. rotundata*, depending on the authors [4–7]. *Dioscorea rotundata* is mainly dioecious, bearing male and female flowers on separate individuals, while some monoecious plants, bearing male and female flowers on the same individual, could be observed [8,9]. It is the most cultivated species in Benin [4], and Benin is the fourth largest producer of this species worldwide after Nigeria, Ghana and Côte d'Ivoire [10]. It is cultivated for tuber production and is an important source of food and income in Benin where yam is the staple food of the population [11]. However, despite its economic importance, this species remains largely disregarded by breeding programs [11]. The species still suffers from phytosanitary problems and is sensitive to environmental stress such as drought or

flooding [12–15]. In sensitive cultivars, anthracnose, fungal or viral attacks may cause yield losses of about 15–25% [14–19].

Even though they are mainly multiplied by clonal propagation, more than 73% of Beninese *D. rotundata* cultivars flower [5] and produce fruits and seeds that allow sexual reproduction [5,20,21]. Although sexual reproduction offers opportunities for genetic improvement, breeders have to face several difficulties inherent to the species, such as a relatively long cultivation cycle (8 to 12 months), low germination and flowering rates, low flower fertility, heteroploidy [8] and especially spontaneous variations in sex from year to year [9]. These spontaneous variations in sex from year to year have not been clearly investigated yet, but they were observed in some cultivars by growers and researchers [9,22]. For example, clonal propagation of a bisexual plant could sometimes result in the next generation giving bisexual, unisexual or non-flowering plants. Similarly, clonal propagation of a unisexual plant may sometimes give unisexual, bisexual or non-flowering plants in the next generation. Spontaneous variations in sex have caught the attention of many researchers since it undermines experimental designs, particularly those involving cross-pollination. This sex variation has also raised the question on the control of sex determination in *D. rotundata* [9,22].

Over the last decades, much research has focused on identifying phenotypic traits (stem and leaf color, presence or absence of wax, stains on stems, leaf shape, etc.) that would make it possible to predict the sex of plants before flowering [23]. Furthermore, correlations between sex and ploidy level were also sought, as the species is characterized by a strong heteroploidy [9,23]. In Benin, cultivars previously described as tetraploid, hexaploid and octoploid for a base chromosome number $X = 10$ [4,6] are actually described as, respectively, diploids, triploids and tetraploids with a base chromosome number $X = 20$ [22–26]. With the development of high-throughput sequencing, the genome of *D. rotundata* has been sequenced, making it possible to identify a sex-linked candidate chromosomal region and to develop genetic markers for sexual identification [22]. Based on a cleaved amplified polymorphic sequence (CAPS, sp1) marker, Tamiru et al. [22] demonstrated that in *D. rotundata* sex determination was under the control of a ZZ/ZW (female heterogametic) system as opposed to the XX/XY (male heterogametic) system described in other *Dioscorea* species such as *D. alata* [27], *D. floribunda* [28] or *D. tokoro* [27]. Tamiru et al. [22] also identified a female specific chromosomal region, which led them to develop the marker sp16 to discriminate male plants from female, monoecious and non-flowering plants [22]. This marker allowed identifying the sex of several *D. rotundata* breeding accessions from the collection of the International Institute of Tropical Agriculture (IITA) in Nigeria [22]. A SuperSAGE transcriptomic study was also undertaken to identify genes linked to flowering and sex in this species and identified candidate genes whose expression differed according to sex [9].

In this context, our study aimed to investigate whether the markers sp16 and sp1 allow sex discrimination in cultivated *D. rotundata* yams from Benin. These markers were tested in 119 yam individuals from 101 different cultivars grown in Benin and compared to the sex observed in the field. The relationship between the level of ploidy, sex and genetic markers was also investigated. We further discussed whether cultivars of the species *D. rotundata* in Benin are regulated by a ZZ/ZW (heterogametic female) or XX/XY (heterogametic male) system based on the sp1 marker.

2. Materials and Methods

2.1. Plant Material

We analyzed 119 yam individuals from 101 different cultivars of *D. rotundata* from the national collection of the Centre de Recherche, de Formation, d'Incubation et d'Innovation pour le Développement Agricole (CREFIISDA, Zogbodomey, Benin, 6°56'–7°08' N, 1°58'–2°24' E). Of the 119 analyzed individuals, 72 were male, 37 were female, 7 were monoecious and 3 were non-flowering based on field observations. As some cultivars have differential sex, individuals of certain cultivars showing different sex based on historical data/personal observations over years and locations were analyzed separately, while only one individual per cultivar was analyzed for cultivars with stable sex

(Table S1). The different cultivars were named according to Dansi et al. [29] to avoid any redundancy due to synonymy. Plants were grown in Benin under field condition at CREFIISDA (Zogbodomey, Benin, 6°56′–7°08′ N, 1°58′–2°24′ E). Sowing was performed on 8 May 2019. For each individual, young healthy leaves were collected for DNA extraction. Leaf harvesting was performed after flowering to clearly identify the sex of each individual.

Cultivars were classified as having stable or unstable sex based on the literature [2,4,20,29,30], previous personal observations made during survey campaigns (2017–2018) and observations made during this study (2019). A cultivar was considered as having unstable sex if the described or observed sex varied across years and/or locations. A cultivar was considered to have stable sex if the sex described or observed was always the same whatever the year and location. The flowering history of the studied cultivars is summarized in Table S1. The ploidy level of the cultivars is also mentioned in Table S1 when available (adapted from Dansi et al. [4], with $X = 20$ [22] instead of $X = 10$ [4]). Dansi et al. [4] determined ploidy level by chromosome counting and flow cytometry.

2.2. DNA Extraction, Amplification of the Genetic Markers and Sequencing

Genomic DNA was extracted from frozen grounded leaves according to Fulton et al. [31]. The concentration and quality of DNA were checked using the NanoDrop ND-1000 (Isogen Life Science, De Meern, The Netherlands). For specific amplification of sp1, sp16 and Dr-Actine [22], we used GoTaq DNA polymerase (Promega, Leiden, The Netherlands), 25 ng of plant DNA as a template and specific primers (Table 1) according to the manufacturer's instructions. After an initial denaturation step at 95 °C for 2 min, each cycle consisted of 20 s at 95 °C, 30 s at an annealing temperature depending on the primer combination and 1 min extension at 72 °C, followed by a final extension of 5 min at 72 °C. The primer pairs, annealing temperature and number of cycles used for each amplification are presented in Table 1. Dr-Actine was used as a positive control (housekeeping gene, used for normalizing the PCR amplifications). PCR amplifications were performed in triplicates. For the sp1 marker, the PCR products were cut with EcoRI (New England Biolabs Inc., Frankfurt, Germany). The PCR products were then resolved on agarose gels.

Table 1. List of primers used in this work.

Marker	Primer Sequences	Tm	Cycles	Size (bp)
Dr-Actine	F: 5'-CAGGGAAAAGATGACCCAAATC-3' R: 5'-CCATCACCAGAATCCAGCAC-3'	55 °C	35	~250 bp
sp1	F: 5'-GATCTGGCTTCCTCCATCTTG-3' R: 5'-GCTTGGGTGGTTAGTTTATTGTTTG-3'	52 °C	40	854 bp
sp16	F: 5'-AATGTGTTTAACAGGGTGAATTC-3' R: 5'-GAATTCAGCCGAATATACTTATTC-3'	52 °C	40	262 bp

Amplified fragments for sp16 were sequenced in five cultivars (two females and three males) in order to compare the nucleotide sequences (three independent sequencings per cultivar). The PCR products were either directly sequenced or sequenced after cloning using the TOPO™ TA Cloning™ Kit for sequencing (Thermo Fischer Scientific, Waltham, MA, USA) and TOP10 chemically competent cells according to the manufacturer's instructions. Transformed colonies were identified by PCR and transformed plasmids were extracted using GeneJET Plasmid Miniprep Kit (Thermo Fischer Scientific, Waltham, MA, USA). PCR products and plasmids were sequenced with Genetic Analyser 3100 (Applied Biosystems, Foster City, CA, USA) using Big Dye Terminator V1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and sp16F and R or T3 and T7 primers. Sequences were aligned using BioEdit 7.2 software.

2.3. Data Analysis

All statistical analyzes were performed with R Software Version 3.6.1 [32]. Descriptive analyzes were performed to associate the sex of the cultivars with the genotypes observed for the markers sp16 and sp1. A bivariate distribution of the genotypes of sp16 and sp1 (before digestion) conditional to the sex allowed us to measure the impact of secondary amplifications generated by sp1 on the sex change in some individuals carrying sp16. Finally, a multiple component analysis (MCA) was performed using the MCA function of the FactoMineR package to evaluate possible associations between the sex of the cultivars and the genotypes obtained for the different markers.

3. Results

3.1. Floral Characteristics of Beninese Yam Cultivars

The 119 investigated individuals were grouped into six categories based on their sexual phenotypes in the field (Table S1). Differences between male, female and monoecious inflorescences are presented in Figure 1. Forty-eight individuals were male and belonged to cultivars that produced only male inflorescences whatever the location and season based on the literature and personal observations (Table S1). We described them as stable or typical males (M). Twenty-four individuals were male and belonged to cultivars with unstable sex, since sex variation was reported for these cultivars based on the literature and personal observations (Table S1). We described them as unstable or atypical (M+) males. Eighteen individuals were female and belonged to cultivars with a stable female sex based on the literature and personal observations (Table S1). We described them as stable or typical females (F). Nineteen individuals were female and belonged to cultivars that changed sex depending on location or season based on the literature and personal observations (Table S1). They were called unstable or atypical females (F+). Seven individuals were monoecious and belonged to cultivars that flowered generally or were occasionally monoecious (O) (Table S1). The last three individuals were non-flowering (NF).

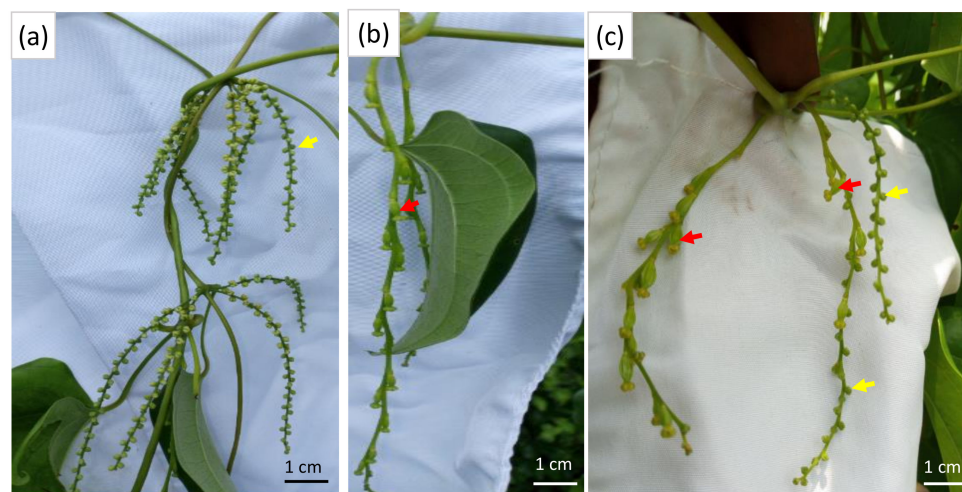


Figure 1. Inflorescences of (a) male, (b) female and (c) monoecious individuals of *D. rotundata* grown under field condition at the Centre de Recherche, de Formation, d’Incubation et d’Innovation pour le Développement Agricole (CREFIISDA) (Zogbodomey, Benin) four months after sowing (three months after tuber germination). Yellow arrows and red arrows indicate, respectively, male and female flowers.

3.2. Use of the Marker sp16 to Discriminate Sex in Beninese Yam Cultivars

According to Tamiru et al. [22], the sp16 marker corresponds to the W allele which is present in female individuals. Our results showed that within cultivars of the species *D. rotundata* from Benin, the marker sp16 amplified mainly in female and monoecious individuals compared to male and

non-flowering individuals (Figure 2a, Table S1). Indeed, 36 of the 37 female individuals (F and F+) amplified the sp16 marker including all the F individuals (Figure 2b). The F+ individual without sp16 amplification belonged to the cultivar Ossousouhoun (syn. Ossoukpana). All monoecious (O) individuals also amplified the sp16 marker (Figure 2b). Thus, the W allele was present in all female (F) and monoecious (O) cultivars studied. For male (M and M+) individuals, 34 out of 72 showed amplification for the sp16 marker (Figure 2b, Table S1), suggesting that 47.22% of male individuals carried the W allele. When considering only male cultivars with stable sex (M), sp16 amplification was observed in 35.42% of the individuals (Figure 2b, Table S1). However, 70.83% of M+ individuals showed amplification for sp16 and carried the W allele.

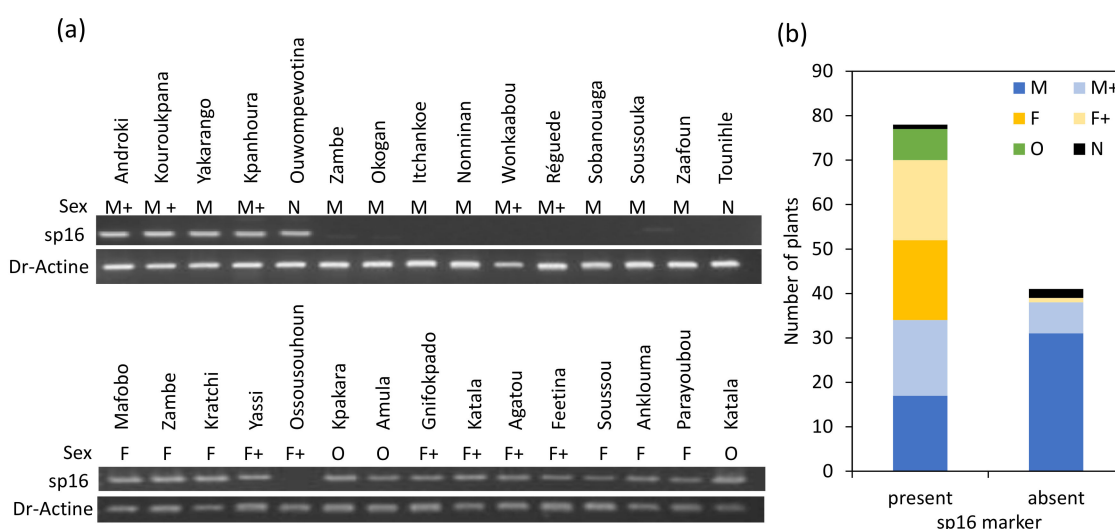


Figure 2. Analysis of the genetic marker sp16 in cultivars of *D. rotundata* from Benin. (a) PCR amplification for sp16 and Dr-Actine in a few cultivars. Dr-Actine is used as a positive control. (b) Distribution of individuals according to their sex identity and the presence or absence of the sp 16 marker. M, stable male; M+, unstable male; F, stable female; F+, unstable female; O, monoecious; and N, non-flowering. The gels with markers are available in the (Supplemental data Figure S2).

The amplified sequences for sp16 were compared between two female and three male individuals from different cultivars, and the nucleotide sequences were identical whatever the sex and the cultivar (Figure S1), suggesting that this fragment of the W allele is the same in female and male individuals, when present.

3.3. Use of the Marker sp1 to Discriminate Sex in Beninese Yam Cultivars

The CAPS marker sp1 was developed by Tamiru et al. [22] and allowed discrimination between homozygous male (ZZ) and heterozygous female (ZW) individuals in *D. rotundata*. Digestion of the sp1 fragment by the EcoRI enzyme showed four different genotypes in cultivated *D. rotundata* yams from Benin, namely AA, AB, BB and AC (Figure 3a,b). AA and BB genotypes were the most frequent and were observed in 60.5% and 31.1% of individuals, respectively, while BB and AC genotypes were observed in only 5.9% and 2.5% of the individuals, respectively. As shown in Figure 3c, 62.16% of female (F and F+) individuals were of AA genotype and 83.33% of male (M and M+) individuals were of AB genotype. The six individuals of genotype BB were male. The AC genotype was rare and distributed among different sexual types (present in one female, two males and two monoecious individuals). We observed that the male sex was more often associated with the genotype AB (or BB but low frequency), while female sex was more often associated with the genotype AA. Nevertheless, the marker sp1 did not allow systematic discrimination between male and female sex in cultivated *D. rotundata* yams from Benin.

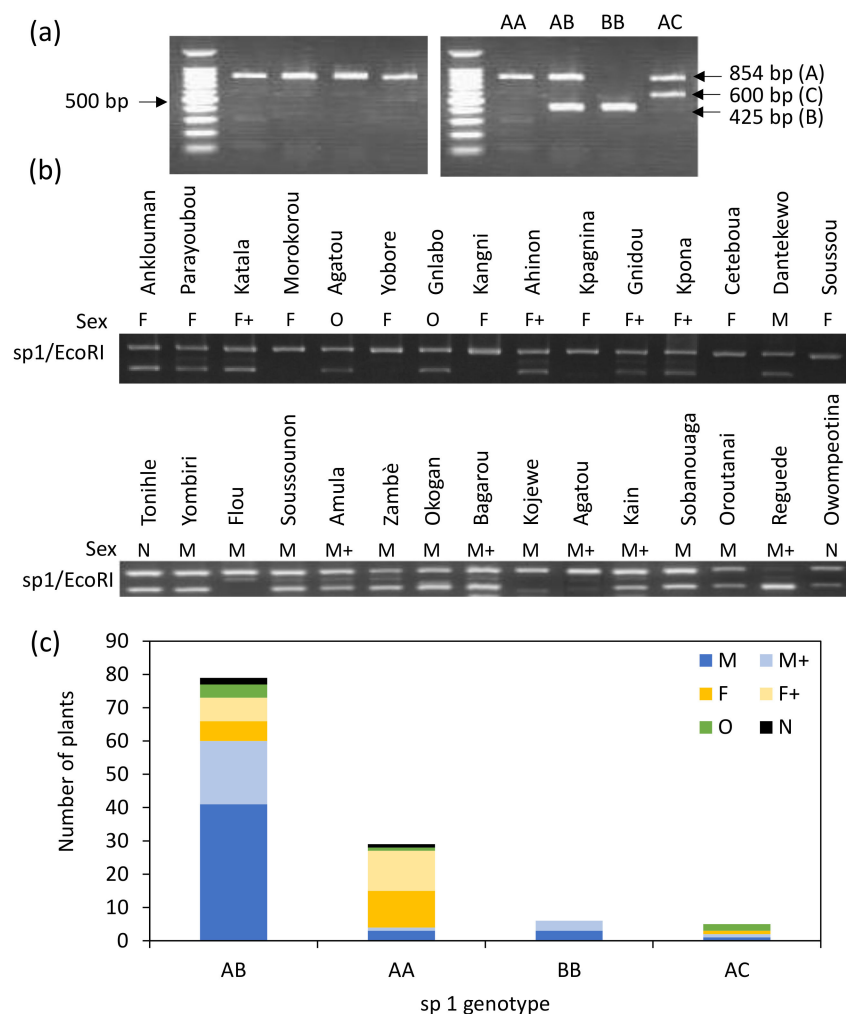


Figure 3. Analysis of the genetic marker sp1 in cultivars of the *D. rotundata* species of Benin. (a) The four profiles obtained before (left) and after (right) restriction with EcoRI. (b) PCR amplification and restriction for sp1 in a few cultivars. (c) Distribution of individuals according to their sex identity and the genotypes (AB, AA, BB, AC) for the sp1 marker. M, stable male; M+, unstable male; F, stable female; F+, unstable female; O, monoecious; and N, non-flowering.

Amplification of the sp1 marker led to secondary amplifications in some cultivars of *D. rotundata* in addition to the 854 bp band that was present in all individuals (Figure 4a). Secondary amplifications of low intensity and variable size (sp1OA) were indeed observed in 60% of studied individuals (Figure 4). The frequency of these secondary amplifications was particularly high in unstable male (M+) and female (F+) individuals which showed a band for the sp16 marker (Figure 4, Table S1). As shown in Table 2, 57.17% of the unstable male (M+) individuals carried the W allele (sp16 marker) and showed sp1OA. Similarly, 63.16% of the unstable female (F+) individuals carried the W allele (sp16 marker) and showed sp1OA. Thus, the combined presence of the W allele and sp1OA could be a sign of sexual instability.

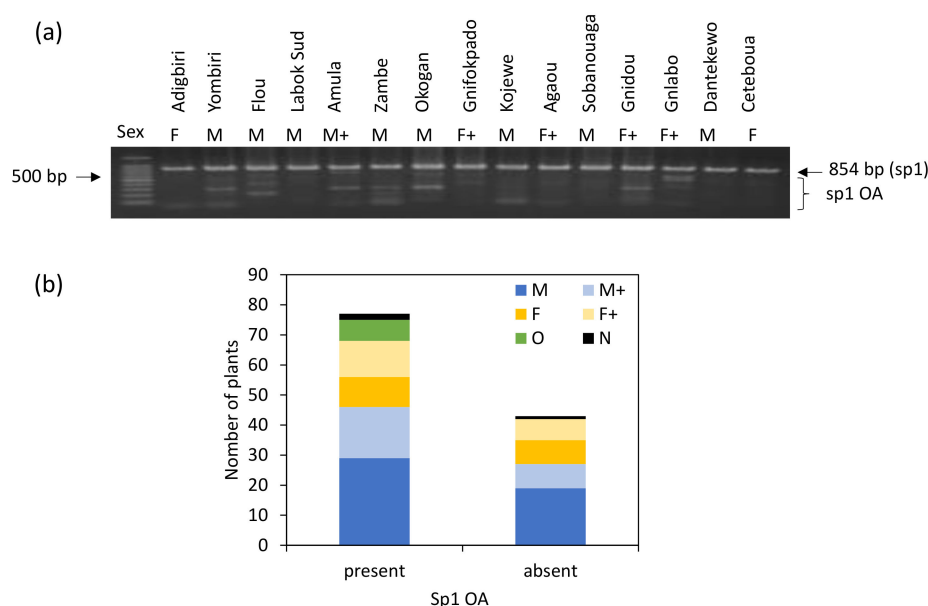


Figure 4. Presence of multiple amplifications for the sp1 marker before restriction in some cultivars of *D. rotundata* from Benin. **(a)** PCR amplification of sp1 before restriction in a few cultivars. **(b)** Distribution of individuals according to their sex identity and the presence or absence of multiple bands for the sp1 marker (sp1 OA) before restriction. M, stable male; M+, unstable male; F, stable female; F+, unstable female; O, monoecious; and N, non-flowering.

Table 2. Impact of the presence of the sp16 marker and the secondary amplifications for the sp1 marker (sp1 OA) on the frequency of unstable male (M+) and female (F+) individuals of *D. rotundata* from Benin.

	Male (M+)		Female (F+)	
	sp1OA absent	sp1OA present	sp1OA absent	sp1OA present
sp16 absent	12.50%	16.67%	5.26%	0.00%
sp16 present	16.67%	57.17%	31.58%	63.16%

3.4. Relative Importance of sp16 and sp1 Markers in Sex Identification in Beninese Cultivars

In order to understand the extent to which each of the two markers explain the sex of *D. rotundata* cultivars from Benin, a multiple component analysis was carried out (Figure 5). The first two dimensions explained 25.7% and 16.4% of the variability, respectively. The first dimension separated male and female individuals according to the sp16 marker. The absence of the sp16 marker was more associated with male individuals and especially with stable male individuals (M) compared to unstable ones (M+). The second dimension distinguished stable male (M) and female (F) individuals from unstable (M+ and F+) individuals based on the presence or absence of sp1 OA. Unstable individuals (F+ and M+) were most often associated with the presence of sp1OA so that, combined with the presence of sp16, secondary amplification at sp1 increased the frequency of male and female cultivars of unstable sex. With respect to the sp1 marker, the AA genotype was more often associated with female individuals while the AB and BB genotypes were more often associated with male individuals. From this analysis, it appears that the best determinant of stable male cultivars is the absence of the sp16 marker and that the best determinant of stable female cultivars is the AA genotype of the sp1 marker.

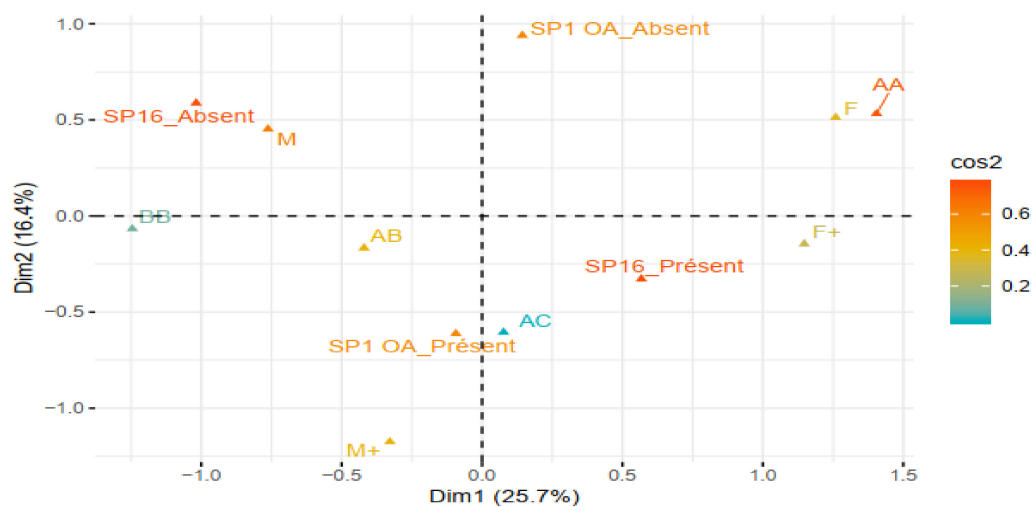


Figure 5. Multiple component analysis (MCA) of the relationship between the sex of *D. rotundata* cultivars from Benin and the markers sp16 and sp1. M, stable male; M+, unstable male; F, stable female; F+, unstable female; O, monoecious; N, non-flowering; and sp1 OA, presence of multiple amplifications for the sp1 marker. The color codes from red to blue (for a cos2 from the highest to the lowest) indicate the decreasing representativeness of the variables.

3.5. Ploidy Level and Sex Expression

The majority of cultivated *D. rotundata* yam individuals analyzed in this study were diploid with a base number $X = 20$ [4,22,23,25,26] (Table S1). Among the 119 individuals analyzed, 2 were triploid and 2 were tetraploid (revised from Dansi [4], Table S1). All individuals tested with a ploidy level greater than $2X$ were male or non-flowering and showed amplification for the marker sp16 and the genotype AB for the sp1 marker (Table 3). Although the number of triploid and tetraploid individuals was too small to assess the interference of ploidy level on the W allele, our results suggest that cultivars with ploidy greater than $2X$ are male or non-flowering despite the presence of the W allele.

Table 3. Impact of polyploidy on sex identity and genotypes for the sp16 and sp1 markers.

Cultivar	Ploidy Level	Sex ¹	sp16 Marker	sp1 Marker
Baridjo	3X	M	Present	AB
Doundoua	4X	M	Present	AB
Owompeotina	3X	N	Present	AB
Alakissa	4X	M	Present	AB

¹ M, male; N, non-flowering.

4. Discussion

4.1. Discriminating Power of the sp16 Marker and Link between the W Allele and Sex Determination

Analysis of our results showed that absence of the sp16 marker could be associated with male identity, but its presence did not systematically imply a specific sex. These results are in agreement with those of Tamiru et al. [22]. Indeed, for Tamiru et al. [22], there is a specific region linked to the female sex characterized by the W allele that is able to partially (partial dominance) or totally (complete dominance) suppress the effect of the male Z allele. These authors hypothesized that this Z-suppressing function can be affected by the environment [22]. As a result, the ZW genotype determines generally female or monoecious identity but may also identify, in some cases, non-flowering phenotypes, while the ZZ genotype always leads to male identity. However, when the Z-suppressor effect of the W allele fails completely, a male individual could be obtained with a ZW genotype [22].

According to these authors, male sex is the default sex and the W allele could be influenced by the environment [22]. The importance of environment on the sexual control of yams has indeed been repeatedly stated [33–36]. However, according to our results, the W allele was present in female individuals but also in more than 42% of the male individuals of *D. rotundata* from Benin. In addition, the sequence of the marker was identical between male and female individuals and therefore did not allow sex discrimination. Thus, the sp16 marker did not allow clear sex discrimination in cultivated white Guinea yam from Benin, while it allowed sex discrimination of improved IITA *Dioscorea rotundata* varieties from Nigeria [22]. If the environment is responsible for modulations of the Z-suppressive effect of the W allele as proposed by Tamiru et al. [22], it would play a significant role in sex determination in *D. rotundata* cultivated yams from Benin. Indeed, some Beninese cultivars change spontaneously from sex identity depending on the year and region as we have observed (Table S1). However, no formal proof of the impact of the environment on the W allele has been obtained yet. Further studies will thus be required to analyze sex-switch from year to year and the impact of the environment on sex determination in *D. rotundata*. Moreover, individuals with different sex were observed for a same cultivar in the frame of this study, although they were cultivated in the same conditions (Table S1). This suggests that sex instability could not only be explained by environmental conditions.

However, given that more than 35% of stable male individuals carried the W allele, we may not exclude that more than one locus are involved in sex determination in white Guinea yam. In their SuperSAGE transcriptome profile, Girma et al. [9] identified 88 tags that were differently expressed between male, female and monoecious individuals. Eighteen of them corresponded to genes known for their involvement in sex determination or floral development in other species [9]. In particular, DnaJ-like protein and Transketolase were specific for sex determination [9]. The latter is located on the chromosomal region linked to the sp1 marker and Z and W alleles [9,22]. On this basis, we cannot exclude that the presumed W allele corresponds to one gene among many others that interact to determine sex expression.

Furthermore, genomic analyzes of Tamiru et al. [22] were carried out on diploid cultivars whereas polyploid cultivars were also observed in *D. rotundata* [9,23]. The ploidy level can also affect sex determination. We observed that individuals with a ploidy level higher than 2X flowered male or were non-flowering despite the presence of the W allele (sp16). Girma et al. [9,23] also observed that cultivars with a high ploidy level were always male or non-flowering. Further investigations are required to understand the interaction between ploidy level and genetic control of sex identity.

4.2. Homo/heterogametic System in *D. rotundata*

The sp1 marker is a CAPS marker associated with a region close to the Z allele, which allowed Tamiru et al. [22] to demonstrate that sex identity of *D. rotundata* is governed by a ZZ/ZW (heterogametic female) system. The use of this marker in our study revealed four genotypes (AA, AB, BB and AC) in cultivated white Guinea yam from Benin with a very low frequency of BB and AC genotypes. These results contrasted with the observations of Tamiru et al. [22]. Such a discrepancy could be explained by the difference of analyzed material. Indeed, Tamiru et al. [22] investigated the progeny of a biparental cross while this study investigated several cultivars. The marker sp1 allowed Tamiru et al. [22] to observe two types of genotypes, one homozygous AA and one heterozygous AB, which respectively corresponded to the male and the female phenotype. Contrary to this description, our results showed that the homozygous AA genotype was rather associated with the female sex and the heterozygous AB genotype with the male sex in the Beninese cultivars of *D. rotundata*. Thus, our results suggested that cultivated white Guinea yam from Benin showed rather a heterogametic male XX/XY system based on sp1 marker analysis. Our hypothesis is in accordance with the genetic control of other yam species. Indeed, according to Martin et al. [28], males are characterized by a heterogametic XY system in the genus *Dioscorea*. The male heterogametic XY system was confirmed in *D. foribunda* [27,28] and in *D. tokoro* [37]. More recently, the heterogametic XY system was also confirmed in *D. alata* [27], a species that belongs to the Enantiophyllum section of the *Dioscorea* genus, such as *D. rotundata*. [27].

Some authors suggested that the presence of a few homozygous males and heterozygous females is due to a transition from a heterogametic male XX/XY system to a heterogametic female ZZ/ZW system [27], while others suggested that it is more the result of rare spontaneous hybridizations that the species has undergone in the past [38,39]. Both hypotheses are plausible in our case. On the one hand, the fact that the sp1 marker co-segregates with the male sex favors the second hypothesis. On the other hand, the presence of the genotypes BB and AC, although very infrequent and of little-known origin to be informative, suggests that there is a beginning of reorganization of the heterogametic XX/XY system within *D. rotundata* from Benin, which thus favors the first hypothesis. In addition, the presence of the W allele (sp16) in 66% of analyzed individuals (including 42% of male individuals) and the fact that the sp16 marker did not allow good sex discrimination in our study for cultivars from Benin also favor the idea of a system in transition in white Guinea yam from Benin. Furthermore, we observed secondary amplifications for the sp1 marker in 60% of the individuals, mainly in cultivars with unstable sex identity. Even if the source of such amplifications is still unknown, they could reflect the strong recombination of this locus and could suggest a process in transition. Tostain et al. [2] investigated the genetic diversity of white Guinea yam cultivars of Benin, including most cultivars analyzed in this study, using simple sequence repeat (SSR) markers. They observed that, in Benin, yam genetic diversity is organized according to cropping regions [2]. They also observed genetic differentiation according to the sex of the cultivars although this relationship requires further investigation. It would be interesting to compare the chromosome organization between lines with stable sexual identity and lines with unstable sexual identity in order to see if the clue of sexual identity is in the chromosome makeup. In the same vein, comparing the sequences of pseudo chromosome 11 in line with stable and unstable sex (F vs. F+ and M vs. M+) could identify key genes and alleles in sexual determination. Our observations and the existence of sexual variations over years and locations in some cultivars suggested that sexual determination is not exclusively under genetic control. It would thus also be necessary to investigate genotype–environment interactions in sex expression and explore whether sex determination of *D. rotundata* could be partly under epigenetic control.

5. Conclusions

After analysis of 119 individuals with sp16 and sp1 markers, our results showed that neither the sp16 nor the sp1 markers allowed a clear sex discrimination in the cultivated *D. rotundata* yams from Benin. We nevertheless observed that the best determinant of stable male cultivars was the absence of the sp16 marker and that the best determinant of stable female cultivars was the AA genotype of the sp1 marker. However, several cultivars had an unstable sex identity. Moreover, high ploidy level was associated with male or non-flowering phenotype, despite the presence of the female W allele. Analysis of the sp1 marker suggested that cultivated white Guinea yam from Benin showed a heterogametic male XX/XY system. However, the variability of our results pushes in favor of the possibility of a reorganization of this system and its transition to a ZZ/ZW system, but this hypothesis remains to be verified. Therefore, further and in-depth studies are required to elucidate the systems governing sexual chromosomes for a better understanding of sex determination in cultivated *D. rotundata* yams.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/10/1521/s1>, Table S1: List of the cultivars analyzed with indication of the sex, flowering history, ploidy level and results for sp16 and sp1 markers. Figure S1. Comparison of the sp16 marker sequence in female and male individuals of *D. rotundata* from Benin. The aligned sequences are consensus sequences of two female and three male individuals from different cultivars. Figure S2. Analysis of the genetic marker sp16 in a few cultivars of *D. rotundata* from Benin. *Dr-Actine* is used as a positive control. These gels are the same as shown in Figure 2. M, stable male; M+, unstable male; F, stable female; F+, unstable female; O, monoecious; and N, non-flowering.

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