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Sample Preservation and Plant Sex Prediction in White Guinea Yam (*Dioscorea rotundata* Poir.)

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Abstract

Introduction: Yam (*Dioscorea* spp.) is an economically important staple food in tropical regions, especially for people in West Africa. Understanding of the flowering behavior of the crop to determine potential manipulation available to accomplish crop improvement at early stage remain key challenge in the yam breeding. The methods that reliably yield quality DNA and distinguishing sex type at the early stage of growth have been a challenge in yam genetics and breeding studies. This study assessed the effect of sample preservation methods on DNA quantity and quality during extraction and potential of DNA marker to diagnose plant sex at the early seedling stage in white Guinea yam.

Materials and Methods: Five sample preservation methods were assessed for quality DNA extraction during field leaf tissue collection, namely liquid nitrogen, dry ice, silica gel, 95% ethanol, and oven drying. The predicted sex at the seedling stage using the molecular marker was further validated with the visual score for the sex phenotype at the flowering stage.

Results: According to the findings of the present study, the DNA extracted from leaf samples preserved in liquid nitrogen, silica gel, dry ice, and oven drying methods were higher in molecular weights than samples stored in ethanol solution. Yam plant sex diagnosis with the DNA marker (sp16) identified a higher proportion of ZW genotypes (female or monoecious phenotypes) than the ZZ genotypes (male phenotypes) in the studied materials with 74% prediction accuracy.

Conclusions: The results from this study provided valuable insights on suitable sample preservation methods for quality DNA extraction and the potential of DNA marker sp16 to predict sex in white Guinea yam.

Keywords: Dioecious, DNA Quality, Flower Type, Sample Preservation Method, Sex Genotype, Sex Phenotype

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Introduction

Yam (*Dioscorea* spp.) is an economically important staple food in tropical regions, especially for people in West Africa.[1,2] It is a preferred staple for over 300 million people in the humid and sub-humid tropics, and the second most important food crop in West Africa.[3] Among the eight primarily cultivated yam species in West and Central Africa, white Guinea yam (*D. rotundata*) is the most preferred for food and livelihood in the region.[4]

Despite its economic and social relevance, yam cultivation is yet to attain its maximum production potential as productivity has remained static for several decades.[4] Genetic improvement through breeding is one of the feasible means to raise the crop's productivity. Flowering of a plant is the basic requirement for any crop breeding effort.[5] The dioecious or monoecious pattern of flowering and sometimes non-flowering is a common phenomenon within and between the *Dioscorea* species. White Guinea yam is mostly dioecious with distinct male or female flower on individual plants.[6] However, in some cases, the yam clone may not flower at all or may express a monoecy, a condition where both male and female flowers occur on the same plant.[4,7] Sex expression and flowering intensity in yam crops are often influenced by growing conditions and the type of propagules used for planting.[6,8,9] Spare flowering pattern, low pollen viability, low stigma receptivity, low fruit set and seed set are key features of the yam crop that often pose challenges for genetic improvement of the crop through conventional breeding.[4,9-12] It is therefore essential that breeders have a good understanding of the flowering behavior of the crop to determine potential manipulation available to accomplish crop improvement.[5]

Identification of sex phenotype in yam crop often requires significant time as it is mostly done when the plant reaches the flowering stage in an environment conducive for flowering.

Different descriptors and marker systems ranging from phenotypic to molecular are often used for flowering and sex type diagnosis in plants.[13-15] The association of flower type with morphological features and ploidy level have been reported in white Guinea yam.[7,12] Triploid yam plants all express either male or non-flowering and have some morphological features distinct from their diploid counterparts.[12] In most of the cases, phenotypic markers used to predict or distinguish sex types are less accurate, delayed in expression, and influenced by growth conditions.[16] Biochemical assay and molecular markers are viable options to accurately predict flowering and sex type in plants at early growth stages.[17,18] The genetics of sex determination systems in white Guinea yam was dissected using whole-genome sequencing and polymerase chain reaction (PCR) primer pair from the SNPs linked to female-specific regions which was developed for sex identification of white Guinea yam at the seedling stage.[6] Similarly, Cormier et al[19] reported the differentiation of flower sex expression in greater yam (*D. alata*) using genotyping-by-sequencing. Genes related to flower development and sex determination are also reported for manipulation of flowering in white Guinea yam.[7] Assessment of yam sexuality at the early development stage prior to flowering is particularly useful in yam breeding as it enables breeders to select appropriate parents for planned and controlled pollination in crossing blocks. Early identification of plant sex type of parents will therefore save labor, time, and cost and improve hybridization efficiency in a breeding program.[20]

Leaf sample collection is a prerequisite for plant sex diagnosis with molecular markers. The leaf samples for molecular analysis are collected from research fields at different locations, including those located at a distance and in remote areas in most of the cases.[21] Getting high-quality biological macromolecules like DNA, which degrade over time, from plant tissue samples collected for experimental purposes is always challenging for many research projects.[22] For quality DNA extraction, it is important to use the best sample preservation method that can maintain DNA integrity for the longest time, especially for plant species whose samples are difficult to collect and large samples that are collected from sites far from laboratories.[21,23] The objectives of this study were therefore, to assess the different leaf sample preservation methods for quality DNA extraction and to explore the potential of DNA marker as a quick method of sex determination prior to flowering in white Guinea yam breeding.

Materials and Methods

The Plant Material

The plant material used in this study consisted of 190 white Guinea yam genotypes ranging from landraces to early generation breeding populations. The materials were sourced from the yam breeding unit of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria (7° 29.46”N, 3° 53.01”E) and were grown at IITA experimental fields in 2017 and 2018 cropping seasons. From among the 190 selected clones, 54 were with known flower sex phenotype from previous field phenotypic data and were deliberately included in the experiment to assess the accuracy of the marker prediction. The rest of the clones (136) were progenies selected from 19 different families originated from bi-parental crossing.

Leaf Sample Preservation Methods for Obtaining Quality DNA During Extraction

Five sample preservation methods were assessed for quality DNA extraction during field leaf tissue collection, namely liquid nitrogen, dry ice, silica gel, 95% ethanol and oven drying. Plant tissue samples were collected from juvenile seedlings in the field at two months after planting when the plants were well established (≥10 leaves stage). Single plant per genotype was tagged with ribbon for the molecular assay and were monitored for sex phenotype at flowering. For liquid nitrogen and dry ice preservation methods, two young leaves were collected from the target plant, placed in well labeled tea bags and samples were stored in liquid nitrogen and dry ice during field collection for a period of 1h and later transferred to -80^{O}C freezer for 72h prior sample lyophilization. Samples were then freeze-dried at -196°C for 96 hours using the Labkoko freeze dry machine. For the samples preserved using silica gel, leaves were placed in 20 g of granular silica gel mixed with color indicator and stored in the dark for 72 hours at room temperature. For each genotype, 2 g of dry leaf was removed from a bag containing the silica gels for DNA extraction. For samples preserved using ethanol (95% v/v) solution before DNA extraction, eight-disc pieces (6-8 mm) were made from young leaves and stored in a 1.5 mL plastic centrifuge tube containing 0.5 mL of 95% ethanol for 72 hours. The sampled discs were then rinsed in deionized water before DNA extraction. For the oven drying method, the leaf samples collected from the field with dry ice were transferred into labeled tea bags and kept in the oven at 45^{o}C for 12 hours. Dried samples were removed and carefully transferred into DNA extraction tube and were immediately subjected to DNA extraction.

DNA Extraction

The DNA was extracted from the leaf samples conserved with different preservative methods using the modified protocol.[24] Genomic DNA was extracted from the leaves using cetyltrimethylammonium bromide (CTAB) procedure. The concentrations and quality of DNA were measured following separation with a 1% agarose gel electrophoresis. The gel picture was captured using a UV light gel documentation system (Aplegen). The DNA concentrations were estimated by measuring the absorbance at 260 nm (A260), 280 nm (A280) and 230 nm (A230) in the Gene Quant pro spectrophotometer (Amersham Bioscience, Piscataway, NJ, USA). The DNA Purity or quality was determined by calculating the ratio of absorbance at 260 nm and 280 nm (A260/A280) as well as 260 nm and 230 nm (A260/230).

Molecular Marker Assay for Flower Sex

The flower sex of the study materials was assayed using two primers: sp16 and D-Actin (Table 1). Marker sp16 is located in a W-linked region and amplifies DNA from female and

Table 1. Primer Sequences Used for Sex Determination in White Guinea Yam

Primer name	Primer sequence (Forward)	Primer sequence (Reverse)
sp16 fragment	5′-AATGTGTTTAACAGGGTGAATTC-3′	5′-GAATTCAGCCGAATATACTTATTC-3′
D-Actin gene fragment	5′-CAGGGAAAAGATGACCCAAATC-3′	5′-CCATCACCAGAATCCAGCAC-3′

monoecious plants.[6] D-Actin served as a PCR (polymerase chain reaction) control to show that DNA template were present in all samples. The DNA sample was diluted to a working solution of 25 ng/µL and was subjected to PCR. Primer optimization was done initially to identify the best annealing temperature. The PCR cocktail had 10 µL of the reagents (Ultra-pure water at 4.34 µL, 10x NH_4 (PCR reaction buffer) at 1µL, 50mM $MgCl_2$ at 0.4 µL, 25mM dNTPs at 0.2 µL, DMSO at 1 µL, 25 ng/µL forward primer at 0.5 µL, 25 ng/µL reverse primer at 0.5 µL, 5 U/mL Taq polymerase at 0.06 µL and 25 ng/µL DNA template at 2 µL). The polymerase chain reaction followed an optimized program with initial denaturation at 94°C for 3 minutes; denaturation at 94°C for 1 min; annealing at 48°C (SP16), 54°C (D-Actin) for 1 minute; extension at 72°C for 1 minute; final extension at 72°C for 10 minutes; and hold at 4°C until the PCR product was removed. Amplification products were analyzed by electrophoresis on a 1.5% agarose gel stained for 3 minutes with Ethidium Bromide and gel was visualized using a UV light gel documentation system (Aplegen). The PCR amplicon/fragment for sp16 marker was scored as present (1) or absent (0). It is said that where the presence of band ranged from 100 to 150 bp is predicted as either female or monoecious flower sex while the absence is predicted as male flower sex.

Visual Assay for Flower Sex Phenotype

The flower sex was visually assessed for flower characteristics at the flowering stage. Plant sex scoring was done using a standard method based on the yam crop ontology (https://www.yambase.org).[25] The flower sex was assessed using a scale of 0–4 where 0 represented for non-flowering, 1 for plants with male flowers, 2 for plants with female flowers, 3 for monoecious (predominantly male flower), and 4 for monoecious (predominantly female flower).

Results and Discussion

Effect of Sample Preservative Methods Prior to Extraction on Quality of DNA From Yam Leaves

Total DNA extracted from leaf samples preserved in liquid nitrogen, silica gel, dry ice and oven drying protocols were higher in quantity compared to the leaf samples preserved in ethanol solution (Figure 1A, Table S1). The five leaf sample preservation methods exhibited widely varying in DNA quality (Figure 1B). The average ratio of absorbance at 260 nm and 280 nm was above 1.8 for samples preserved with liquid nitrogen, silica gel, dry ice and oven drying methods compared to ethanol as a preservative solution with a quality ratio of 1.63. However, the average ratio of absorbance at 260 nm and 230 nm, as the second measure of DNA purity, was higher than the respective $A_{260/280}$ values for all the sample preservation methods assessed. Surprisingly, the $A_{260/230}$ ratio of samples preserved using ethanol solution was quite higher and in acceptable DNA purity range of 2.0 to 2.2. The molecular weight and quality of the genomic DNA is imperative for reliability, feasibility and reproducibility of molecular genetic studies.[26] The DNA quality analysis follows the principle of absorbance in which the ratios of $A_{260/280}$ and $A_{260/230}$ with a value of approximately 1.8 and 2.0-2.2, respectively is generally accepted as "pure" DNA sample.[27] Samples stored in ethanol resulted in low molecular weight DNA and resultant $A_{260/280}$ ratio suggesting that ethanol is not a suitable preservative solution for quantity-quality DNA extraction from white Guinea yam leaves. Ethanol solution was also reported as an unsuitable preservation method for collecting cassava leaf samples.[21] In contrary to yam and cassava, ethanol solution was reported as a suitable preservation method for leaf samples from *Jatropha curcas* and other tropical species for the extraction of high quality DNA.[28] Several studies reported that method of leaf tissue sampling and preservation contributes to the quantity-quality

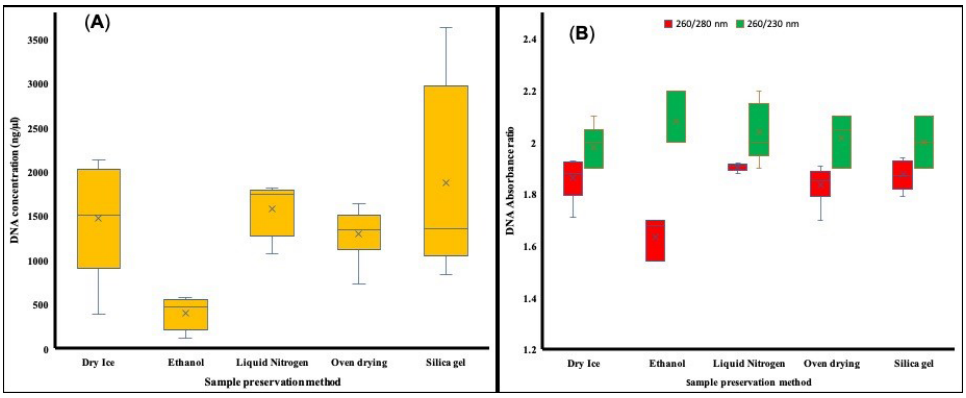

Figure 1. Concentrations (**A**) and Quality (**B**) of DNA Extracted From White Guinea Yam Leaf Samples Preserved Using 5 Different Methods. The DNA purity was assessed based on ratios of absorbance at $A_{260/280}$ and $A2_{60/230}$ nm.

of DNA during extraction.[29-31] Leaf samples are usually dried with a lyophilizing machine which dries biological materials at very low temperature in order to avoid DNA denaturation before extraction. The quality and quantity of DNA extracted from oven-dried leaf samples was high in this study. This, therefore, suggests the suitability of over-drying for white Guinea yam leaf sample preservation for quality DNA extraction in the absence of lyophilizing machine. Oven drying was also reported by Liu et al[32] as a suitable method to obtain quality and quantity DNA for subsequent molecular analysis or genotyping.

Plant Sex Type Assay With Molecular Marker

The PCR amplifications of both the sp16 and D-actin markers on study materials are shown in Figure 2. D-actin is the control and is amplified on all accessions while the sp16 only amplified on some materials with female or monoecious sex phenotype expression.

One hundred and thirty-six progenies obtained from the artificial hand-pollination did not flower in the first year of evaluation (seed progeny), hence, the predicted sex type could not have been confirmed. However, the second-year field monitoring provided a better chance to assess flower sex phenotype expression on both the new progenies (136) raised from botanical seeds and well-characterized clones (54) with known flower sex type. Non-flowering of seed progenies (plants grown from botanical seeds) is a regular phenomenon in white Guinea yam, but flowering improves with consecutive clonal-derivative generations. Tamiru et al[6] monitored flowering in 249 offspring from Guinea yam bi-parental crosses in two planting seasons and reported non-flowering of the seed progenies till the first clonal generation after propagation from the tubers. Similarly, low flowering at seed progeny stage was noticed in two bi-parental populations used for linkage analysis in *D. alata* suggesting that the observed non-flowering at seed progeny stage in this study is a general manifestation of flowering pattern in yam crop.[19]

Among the 54 clones with a prior sex phenotype information, 22 clones had female phenotype, 25 clones had male phenotype, 4 clones had monecious phenotype with male predominance, and 3 clones had monoecious phenotype with female type prevalence. In the second season, flowering was noticed on 102 out of the 190 genotypes. Among the genotypes that flowered, 30 had female phenotype, 64 had male phenotype, 5 had monecious phenotype with male prevalence, and 3 clones had monoecious phenotype with female predominance. Sixty-nine progenies from bi-parental crosses did not flower at the seed progeny and consecutive tuber progeny stages. Nineteen progenies failed to establish in the field at the first clonal generation stage. The flowering pattern and flower sex types observed among the materials used for this study indicated the predominance of dioecious and shy to flower pattern with a few monoecious in the white Guinea yam (Table 2 and Figure 3). The segregation for flower sex in the studied materials was 18% female, 37% male, 5% monoecious and 40% non-flowering. Flower sex segregation was 12% female, 35% male, 10% monoecious and 43% non-flowering in the source population (367 offspring derived from eight half-sib families) from where the materials used for this study was sampled (data not shown).

The four sex phenotypes observed among the materials in this study were grouped as two sex genotypes (Table 2) with molecular marker diagnosis. A previous study showed that white Guinea yam has a female heterogametic sex determination system with ZZ=male, ZW=female or monoecious.[6] Female-specific genomic fragment corresponding to the W-region spans at least ~160 kb and sp16 marker is located on it. The grouping of the observed four sex phenotypes to two sex genotypes in the present study confirmed the sp16 as a female-specific marker which only amplifies on genotypes with W-locus. Sex expression in white Guinea yam is determined by Z and W locus segregation with the homozygous locus state ZZ represents the male phenotype while the heterozygous (ZW) or hemizygous (Z-) locus state represents the female phenotypes and plants with monoecious or male phenotype depending on growth environment.[6] An unstable sex prediction was observed in the current study which was predicted by the marker as female but classified as a male or monoecious individual based on visual flower characteristic assay. Clones with monoecious phenotype

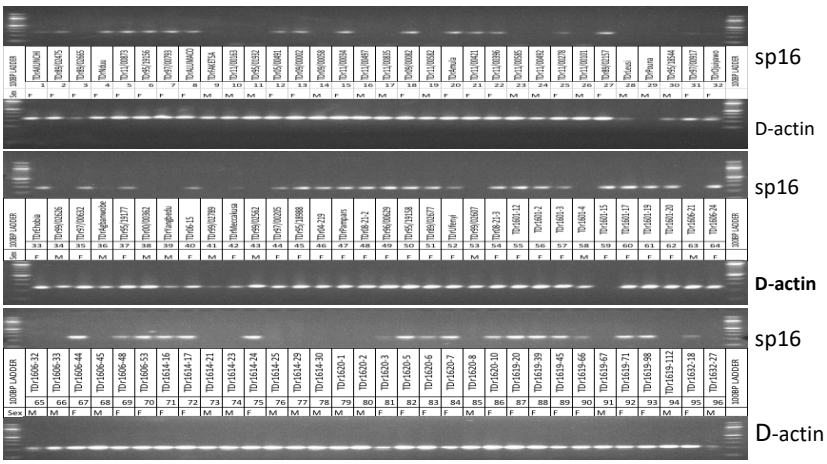

Figure 2. PCR Amplification for Both sp16 and D-actin Markers.

Table 2. Summary of the Sex Determination Via Phenotyping and Genotyping of 190 White Guinea Yam Clones in 2017 and 2018 Planting Seasons

Sex Type	2017 Season	2018 Season	Sex Genotype
Female	22	30	120
Male	25	64	70
Monoecious-Female	3	3	
Monoecious-Male	4	5	
Non-flowered	136 seed progenies	69 tuber progenies	
Not survived	0	19	
Total	190	190	190

Marker prediction accuracy (%) is 73.53% in total flowered phenotypes and 83% in clones with well-known sex phenotype

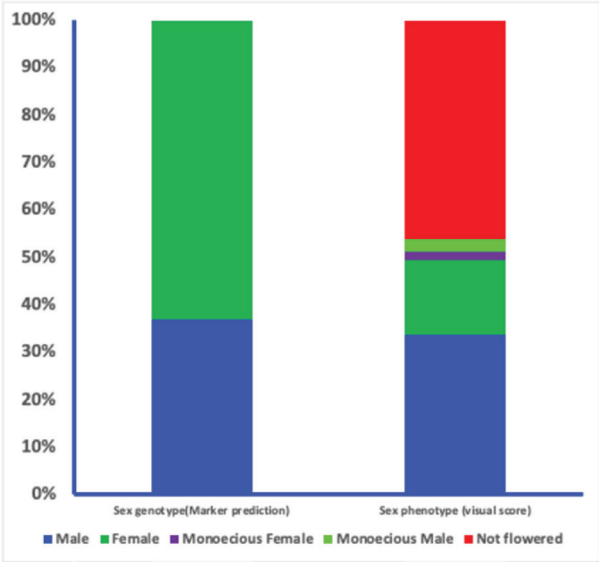

Figure 3. Sex distribution among 190 white Guinea yam genotypes analyzed using sp16 marker and visual flower characteristic assay. The percentage of sex genotype was based on the sex prediction using sp16 marker in all plants including non-flowering plants.

(male and female flowers appear on the same plant) was considered as a female genotype by sp16 marker assay due to the presence of W-allele in hemizygous status. The sp16 marker assay at earlier vegetative seedling stage suggested 63% (120 of the 190 accessions) as female genotype (ZW or Z-) and 37% (70 clones) as male genotype (ZZ) (Table 1 and Figure 3, Table S2). The yam plant sex prediction accuracy with sp16 marker was 83% of the cases with the 54 clones having well-known flower sex phenotype and 74% of the cases among the total genotypes produced flower during the field phenotyping (Table 2). The sp16 marker accurately identified the sex phenotype of all well characterized clones except eight males and two females (Table S2). The male phenotype diagnosed as female with sp16 marker were Alumaco, TDr11/00034, TDr09/00082, TDr11/00421, TDr11/00396, Ehobia, TDr06-15, and TDr89/02677 whereas the female phenotype distinguished as male with the sp16 marker were TDr11/00835 and TDr95/18544. The amplification of eight male phenotype with sp16 marker could be a result of monoecious phenomenal observation in yam with complete lack of expression of female flowers in these 8 genotypes. The two female phenotype clones that did not amplify could be as a result of recombination between the marker and the gene or mislabeling during sample collection, DNA extraction, storage or PCR process.

Furthermore, eight clones (TDr05/00491, TDr09/00002, Amula, TDr11/00278, TDr95/19177, TDr04-219, TDr96/00629, and TDr1619-66) identified as female genotypes by sp16 marker turned out to be monoecious (Table S1). Tamiru et al[6] suggested maleness as a default phenotype and that the W-allele is dominant over Z-allele thus resulting in the feminization of flowers. If the feminizing function of the W-allele fails (differential allelic expression) in a subset of flowers, the individual becomes monoecious. This explains the basis for the behavior of sp16 marker.

In this study, clones with monoecious flower expression were amplified by sp16 marker thus designating them as genotype with ZW alleles. The ZZ genotype consistently gave rise to the male phenotype while the ZW genotype resulted in changes in the sex phenotypes. The ZW genotype is capable of being expressed as female, male and monoecious phenotypes depending on the environment.[6] A similar trend was observed in this study as ZZ genotype consistently produced male flowers (male phenotype) while ZW genotype produced both female and monecious phenotypes and can be attributed to the differential allelic expression of the W-allele.

The observed higher ratio of ZW genotype relative to the ZZ genotype with sp16 marker in the current set of materials used in this study disagrees with the findings of Zoundjihekpon et al[33] who stated that yams grown from botanical seeds have almost equal number of male and female phenotypes. The higher frequency of ZW genotype prediction in the population used for this study suggests the segregation of more female plants from controlled crosses. Availability of more fertile female clones in a breeding program is valuable for exploiting potential of half-sib breeding via open natural pollination which is a convenient and cost-effective strategy to generate larger numbers of seedlings for selection.[9] Female yam phenotypes have been reported to superior agronomic traits such as uniform and early sprouting, better crop establishment, good vigor, higher survival rate and higher tuber yield than male phenotypes.[34,35]

Conclusions

Determining the best leaf sample preservation method for quality DNA extraction is useful for genetic and breeding studies in yam improvement, especially for large samples that are collected from trial sites far from laboratories. Knowledge of sex expression and flowering time is also valuable for more successful hybridization in white Guinea yam. In this study, it was found that liquid nitrogen, silica gel and dry ice are all suitable methods for preserving leaf samples to obtain high quantity-quality DNA in white Guinea yam. In the absence of a lyophilizing machine, oven-drying at 45°C is a good substitute for leaf preservation before DNA extraction.

Finally, it was confirmed that the sp16 marker could be used for early identification of plant sex in yam crossing blocks thereby saving space, time, and labor required in the planning and management of crossing blocks. This will help in shortening the yam breeding cycle. However, further investigation should be carried out to resolve the inability of marker sp16 to discriminate among monoecious, female and non-flowering phenotypes. Identification of more markers accurately locating QTLs or genes controlling phenotypic variation in plant sex type will improve the accuracy and efficiency in yam breeding.

Authors' Contributions

DD conceived the study, AA developed breeding population, CN, PA conducted field and molecular assessments. PA and AA wrote the manuscript, DD and PA contributed to writing. All authors read and approved the final manuscript.

Conflict of Interest Disclosures

The authors declare no conflict of interest.

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Supplementary Materials

The following is available as annex to the main text:

Table S1: Comparison between DNA quantity and quality on five different leaf sample preservation methods for DNA extraction in white Guinea yam.

Table S2: Comparison between phenotypic flower expression and marker prediction of190 white Guinea yam clones in 2017 and 2018 planting seasons.

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