 doi:10.20944/preprints202002.0187.v1

*Article*

# Sample Preservation and Plant Sex Prediction in white Guinea yam (*Dioscorea rotundata* Poir.) Seedlings

**Asrat Asfaw [1,*], Paterne Agre [1], Chidinma Nwachukwu [2], Bunmi Olasanmi [2], Jude Obidiegwu [3], Emmanuel Nwachukwu [3], Patrick Adebola [4] and David De Koeyer [5]**

[1] International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria
[2] Department of Agronomy, University of Ibadan, Ibadan, Nigeria
[3] National Root Crops Research Institute, Umudike, Abia State, Nigeria
[4] International Institute of Tropical Agriculture (IITA), Abuja, Nigeria
[5] Agriculture and Agri-Food Canada, Fredericton, New Brunswick, Canada
* Correspondence: a.amele@cgiar.org; Tel.: +234 8039784287

**Abstract:** Methods for high-quality DNA extraction and knowledge of sex expression and flowering time are essential for applying genomic-assisted breeding and improve the success with hybridization in Guinea yam. A dioecious or monoecious pattern of flowering and sometimes non-flowering is a common phenomenon within and between the Dioscorea species. The flowering in yam plants raised from botanical seeds often takes an extended period, mostly till the first clonal generation after propagation from the tubers. The prolonged process of testing required to identify plant sex and flowering intensity in yam breeding often poses a challenge to realize reduced breeding cycle and apply genomic selection. This study assessed sample preservation methods for DNA quality during extraction and potential of DNA marker to diagnose plant sex at the early seedling stage in white Guinea yam. The predicted sex at the seedling stage was further validated with the visual score for the sex phenotype at the flowering stage. DNA extracted from leaf samples preserved in liquid nitrogen, silica gel, dry ice, and oven drying methods was similar in quality with a high molecular weight 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 phenotype) in the studied materials with 74% prediction accuracy. 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; visual assay

## 1. 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]. Of the eight primarily cultivated yam species in West and Central Africa, *D. rotundata* (white Guinea yam) 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. Genetic improvement through breeding is one of the feasible means to raise the crop's productivity. It is therefore essential that breeders have a good understanding of the mode of flowering of a crop to determine potential manipulation available to accomplish crop improvement

 doi:10.20944/preprints202002.0187.v1



[5]. The dioecious or monoecious pattern of flowering and sometime 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].

Identification of sex phenotype in yam crop often requires significant time as it 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 *D.rotundata* [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 stage [17,18]. [6] dissected the genetics of sex determination systems in white Guinea yam using whole-genome sequencing also developed PCR (polymerase chain reaction) primer pair from the SNPs linked to female-specific regions for sex identification of Guinea yam at the seedling stage. Similarly, [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 identification 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]. Sample preservation method that maintain DNA integrity for the longest time, especially for plant species difficult to collect samples and large sample collections from sites distance from laboratories [21,23]. It is, therefore, of special importance to determine the best method for leaf sample preservation for quality DNA extraction, which have not been adequately investigated for yam. The objectives of this study were to assess the different leaf sample preservation methods for quality DNA extraction and explore the potential of DNA marker as a quick method of sex determination prior to flowering in white Guinea yam breeding.

## 2. Results and Discussion

### *2.1. 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 quality with brighter DNA bands visualized by gel electrophoresis than the DNA preserved in ethanol solution (Figure 1). The DNA quality ratio 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 1.4 (Figure 2). DNA quantity was higher with other methods than the ethanol solution for fixation and preservation of DNA from yam leaves. The molecular weight and quality of the genomic DNA is imperative for reliability, feasibility and

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 14 February 2020 doi:10.20944/preprints202002.0187.v1



reproducibility of molecular genetic studies [24]. The DNA quality analysis follows the principle of absorbance in which the ratio of absorbance at 260 and 280 nm (A260/280) with a value of approximately 1.8 is generally accepted as "pure" DNA samples [25]. Samples stored in ethanol resulted in low molecular weight DNA suggesting ethanol as not a suitable preservative solution for quality DNA extraction from yam leaves. Ethanol solution was also reported as unsuitable preservation method for collection of cassava samples [21]. In contrary to yam and cassava, ethanol solution was reported as a suitable preservation method to leaf samples from *Jatropha curcas* and other tropical species for the extraction of high quality DNA [26]. Number of studies report method of leaf tissue sampling and preservation contributes to the quality of DNA during extraction [27–29]. The high DNA quality and quantity extracted from oven-dried leaf samples in this study, suggested that this method is suitable for drying yam leaf samples without denaturing the DNA in the absence of a freeze dryer. [30] also reported oven drying as a suitable method to obtain quality and quantity DNA for subsequent molecular analysis or genotyping. 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. This study, however, suggest the possibility of obtaining high quality DNA from oven-dried yam leaf samples in the absence of lymphosizing machine which is often not available in molecular laboratories in Africa especially in the national breeding program.

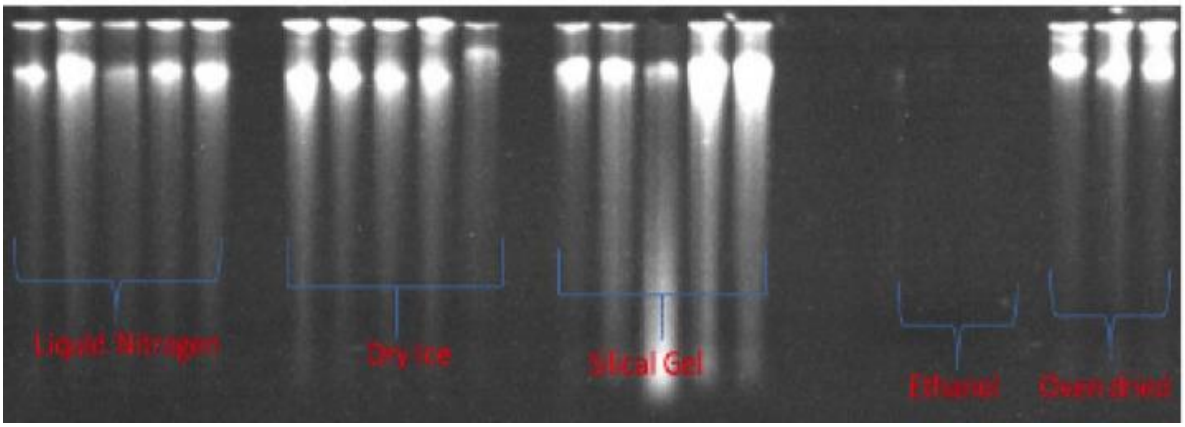


**Figure 1.** Agarose gel showing bands of DNA extracted from leaf tissues collected using five sample preservation methods.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 14 February 2020 doi:10.20944/preprints202002.0187.v1



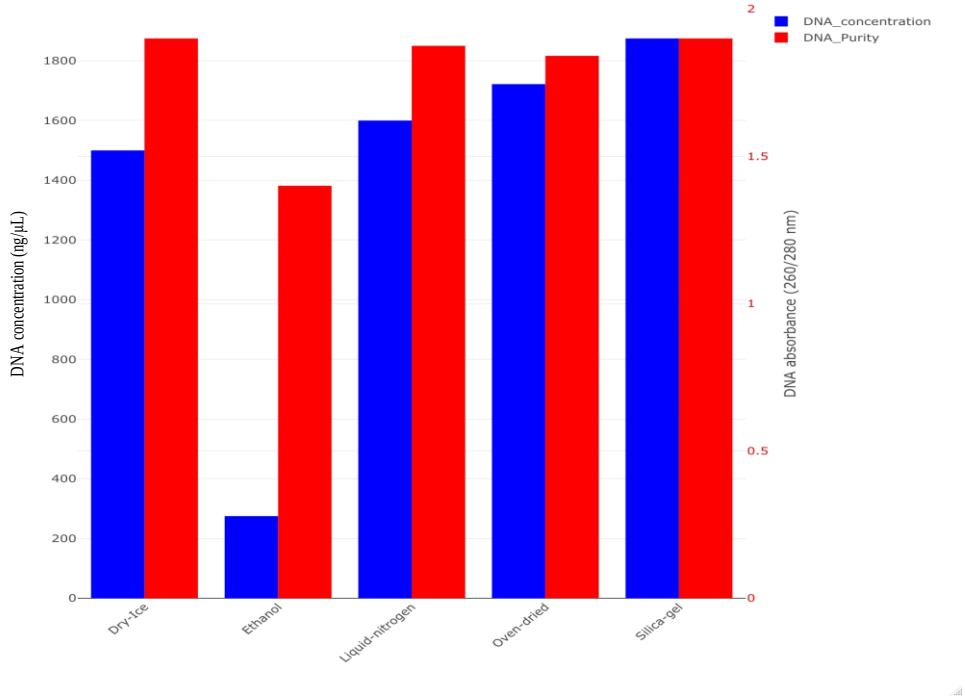


**Figure 2.** Concentrations and quality of DNA extracted from collected leaf tissues using five different leaf sample preservation methods.

*2.2. Plant Sex Type Assay with Molecular Marker*

Figure 3 presents PCR amplification of both the sp16 and D-actin markers on study materials. D-actin is the control and amplified on all accessions while the sp16 only amplified on some materials with female or monoecious sex phenotype expression.


**Figure 3.** PCR amplification for both sp16 and D-actin markers.

One hundred and thirty-six seedling progenies obtained from the artificial hand-pollination did not flower in the first year of evaluation and we unable to confirm the predicted sex type. 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 plants grown from botanical seeds is a regular

 doi:10.20944/preprints202002.0187.v1



phenomenon in *D. rotundata*, but flowering improves with consecutive clonal-derivative generations. [6] monitored flowering in 249 offspring from Guinea yam bi-parental crosses in two planting seasons and reported non-flowering of the seedling progenies raised from botanical seeds till the first clonal generation after propagation from the tubers. Similarly, low flowering of seedling progenies was noticed in two bi-parental populations used for linkage analysis in *D. alata* [19] confirming that the observed non-flowering of first seedling progenies in this study is a general occurrence in yam.

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 flowered genotypes, 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 didn't flower at the seedling progeny and consecutive seed-tuber progeny stages. Nineteen seed-tuber progenies failed to establish in the field at the first clonal generation. The flowering pattern and flower sex types in 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 1 and Figure 4).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 14 February 2020 doi:10.20944/preprints202002.0187.v1



**Table 1.** Summary of the sex determination via phenotyping and genotyping of one hundred and ninety (190) *D. rotundata* clones in 2017 and 2018 planting seasons.

| Sex type | Sex phenotype | | Sex genotype |
|---|---|---|---|
| | 2017 Season | 2018 Season | |
| Female | 22 | 30 | 120 |
| Male | 25 | 64 | 70 |
| Monoecious-Female | 3 | 3 | |
| Monoecious-Male | 4 | 5 | |
| Non-flowered | 136 seedling progenies | 69 seed-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.

The four groups of the observed sex phenotypes in current set of materials were collapsed into two sex genotype groups (Table 1) with molecular marker diagnosis. Previous study showed that *D. rotundata* 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 collapsing of the observed four sex phenotypes to two sex genotypes in our study confirmed the sp16 as a female-specific marker which only amplifies on genotypes with W-locus. Sex expression in Guinea yam is determined by Z and W locus segregation with the homozygous locus state ZZ determines the male phenotype while the heterozygous (ZW) or hemizygous (Z-) locus state determines the female phenotypes and plants with monoecious or male phenotype depending on growth environment [6]. We noticed unstable sex prediction in our current study in some individuals with a marker predicted female sex that appeared to be male or monoecious phenotype with visual flower characteristic assay. Clones with monoecious phenotype (male and female flowers appear on the same plant) was considered as a female genotype by sp16 marker assay due to 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, supplementary Table 1). The yam plant sex prediction accuracy with sp16 marker was 83% of the cases with the 54 clones with well-known flower sex phenotype and 74% of the cases among the total genotypes produced flower during the field phenotyping (Table 1). The sp16 marker accurately identified the sex phenotype of all well characterized clones except the eight males and two females (Supplementary Table 1). 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 as a result of either the marker (sp16) is not completely linked to the gene controlling flower sex or the clones are monoecious plants with complete lack of expression of female flowers. The two female phenotype clones did not amplify could be as a result of recombination between marker and gene or mislabeling during sample collection, DNA extraction, storage or PCR process.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 14 February 2020 doi:10.20944/preprints202002.0187.v1



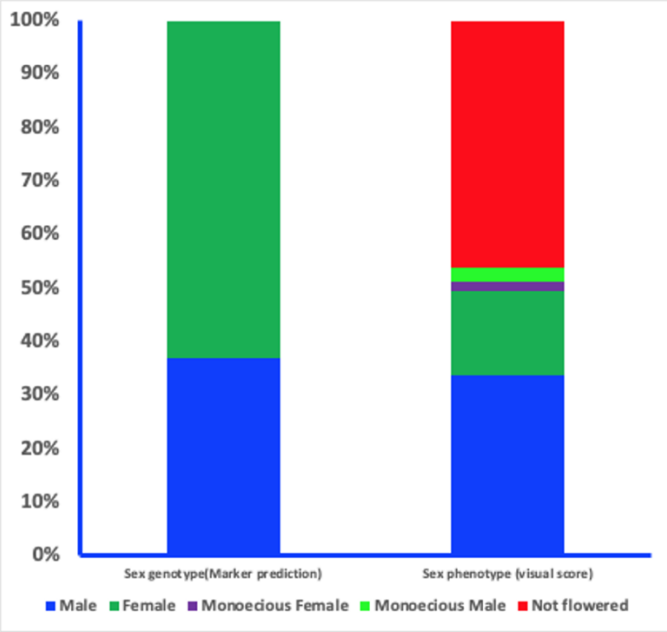


**Figure 4.** Sex distribution among 190 yam genotypes analyzed using sp16 marker and visual flower characteristic assay. The percentage of sex genotype was based on the sex prediction using sp16 marker in all plants including non-flowering plants.

Furthermore, eight clones (TDr09/00002, Amula, TDr11/00278, TDr96/00629, TDr95/19177 and TDr1619-66) identified as female genotypes by sp16 marker turned out to be all monoecious (Supplementary Table 1). [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 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 been expressed as female, male and monoecious phenotypes depending on the environment [6]. 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 diagnosis of higher ratio of ZW genotype relative to the ZZ genotype with sp16 markers in current set of materials disagrees with the findings of [31] 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 the 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 number of seedlings for selection [9]. Female yam phenotypes have been reported to have superior agronomic traits than male phenotypes including uniform and early sprouting, better crop establishment, good vigor, higher survival rate and higher yield [32,33].

## 3. Materials and Methods

### *3.1. The Plant Material*

The plant material used in this study consisted of 190 *D. rotundata* genotypes ranging from landraces to early generation breeding populations. The materials were sourced from the yam

 doi:10.20944/preprints202002.0187.v1



breeding unit at 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. Of the 190 selected clones, fifty-four (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. While the rest 136 were progenies selected from19 different families originated from bi-parental crossing.

*3.2. Leaf Sample Preservation Methods for DNA Quality during Extraction*

Five sample preservation methods during field leaf tissue collection, namely liquid nitrogen, dry ice, silica gel, 95% ethanol, and oven drying, were assessed for DNA quality during extraction. 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 monitoring the flower 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 $^{O}$C for 96 h using Labkoko freeze dry machine. For the silica gel collection, sampled leaves were placed in 20 g of granular silica gel mixed with color indicator and stored in the dark for 72 h at room temperature. For each genotype, an amount of 2 g dried leaf was then removed from the silica for DNA extraction. Absolute ethanol (95% v/v) were used as a preservative solution before DNA extraction, eight-disc pieces (6-8mm) were made from young leaves and stored in a 1.5 mL plastic centrifuge tube containing 0.5 mL of 95% ethanol for 72 h. 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 h. Dried samples were removed and carefully transferred into DNA extraction tube and were immediately subjected to DNA extraction.

*3.3. DNA Extraction*

DNA was extracted from the leaf samples conserved with different preservative methods using the modified [34] protocol. 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, and a gel picture was captured using a UV light gel documentation system (Aplegen). The DNA concentrations were estimated by measuring the absorbance at 260nm (A260) and 280nm (A280) in the Gene Quant pro spectrophotometer (Amersham Bioscience, Piscataway, NJ, USA). DNA Purity or quality was determined by calculating the ratio of absorbance at 260 nm and absorbance at 280 nm (A260/A280).

*3.4. Molecular Marker Assay for Flower Sex*

The flower sex of the study materials was assayed using two primers: Sp16 and Dr-Actin (Table 2). Sp16 is located in a W-linked region and amplifies DNA from female and monoecious plants [6]. Dr-Actin served as a PCR (polymerase chain reaction) control to show that the DNA template present for all samples. DNA was diluted to a working solution of 25 ng/µL and was subjected to PCR reaction. Primer optimization was done initially to identify the best annealing temperature. The PCR cocktail had 10µL of the reagents (Ultra-pure water at 4.34µL, 10x $NH_4$ (PCR reaction buffer) at 1µL, 50mM $MgCl_2$ at 0.4µL, 25mM dNTPs at 0.2µL, DMSO at 1µL, 25ng/µL forward primer at 0.5µL, 25ng/µL reverse primer at 0.5µL, 5 U/mL

Taq polymerase at 0.06µL and 25ng/µL DNA template at 2µL). The polymerase chain reaction followed an optimized program with initial denaturation at 94$^{o}$C for 3 min; denaturation at 94$^{o}$C for 1 min; annealing at 48$^{o}$C (SP16), 54$^{o}$C (Dr-Actin) for 1 min; extension at 72$^{o}$C for 1 min; final extension at 72$^{o}$C for 10 min; and hold at 4$^{o}$C until the PCR product was removed. Amplification products were

 doi:10.20944/preprints202002.0187.v1



analyzed by electrophoresis on a 1.5% agarose gel stained for 3 min 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), 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.

**Table 2.** Primer sequences used for sex determination in *D. rotundata.*

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

*3.5. Visual Assay for Flower Sex Phenotype*

The flower sex phenotype 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 (Asfaw, 2016) (www.yambase.org). The flower sex was assessed using a scale of 0–4 where 0 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).

## 5. Conclusions

Leaf sample preservation methods for high quality DNA extraction and knowledge of sex expression and flowering time in Guinea yam is important for applying genomic-assisted breeding and improved success with hybridization. In this study, we found that liquid nitrogen, silica gel, and dry ice are the most suitable methods for preserving leaf samples for high DNA quality in white yam. We also demonstrated that in the absence of a lyophilizing machine, oven-drying at 45°C is a good substitute for leaf preservation before DNA extraction. Finally, we confirmed that the sp16 marker could be used for early identification of plant sex in yam crossing block potentially saving space, time, and labor required in the design of crossing plan, hence, contribute to shortening the yam breeding-cycle. However, additional research is needed to solve the bias with the sp16 marker to differentiate between monoecious, female and non-flowering phenotypes. Identification of more markers accurately locating QTLs or genes controlling sex phenotype alongside with its function would also improve accuracy and efficiency in yam breeding.

**Supplementary Material:** The following is available as annex to the main text. **Table S1:** Comparison between phenotypic flower expression and marker prediction of one hundred and ninety (190) *D. rotundata* clones in 2017 and 2018 planting seasons.

**Author Contributions:** DD conceived the study, AA developed breeding population, CN, PA[1] conducted field and molecular assessments. PA[1] and AA wrote the manuscript, DD and PA[4] contributed to writing. All authors read and approved the manuscript.

**Funding:** This research was funded by research grant (OPP1052998) to IITA from the Bill and Melinda Gates Foundation.

**Acknowledgments:** We acknowledge funding from IITA through a research grant (OPP1052998) received from the Bill and Melinda Gates Foundation. We thank IITA Bioscience center especially Queen Obi for support in the molecular work. We thank IITA yam breeding team specifically Adewumi Adeyinka, Idris Adejumobi, Prince Norman, Alex Edemodu, Alice Adenike Olatunji, and Kwabena Darkwa who were involved in trial management and field data collection.

**Conflicts of Interest:** The authors declare no conflict of interest

## References


1. Bhandari, M.R.; Kasai, T.; Kawabata, J. Nutritional evaluation of wild yam (Dioscorea spp.) tubers of Nepal. *Food Chem.* **2003**, *82*, 619—623.
2. Loko, Y.L.; Agre, P.; Orobiyi, A.; Dossou-Aminon, I.; Roisin, Y.; Tamo, M.; Dansi, A. Farmers' knowledge and perceptions of termites as pests of yam (Dioscorea spp.) in Central Benin. *Int. J. Pest Manag.* **2016**, *62*, 75–84.
3. Alabi, T.R.; Adebola, P.O.; Asfaw, A.; De Koeyer, D.; Lopez-Montes, A.; Asiedu, R. Spatial Multivariate Cluster Analysis for Defining Target Population of Environments in West Africa for Yam Breeding. *Int. J. Appl. Geospatial Res.* **2019**, *10*, 1–30.
4. Darkwa, K.; Olasanmi, B.; Asiedu, R.; Asfaw, A. Review of empirical and emerging breeding methods and tools for yam (Dioscorea spp.) improvement: Status and prospects. *Plant Breed.* **2019**, 1–24.
5. Fryxell, P.A. Mode of reproduction of higher plants. *Bot. Rev.* **1957**, *23*, 135–233.
6. Tamiru, M.; Natsume, S.; Takagi, H.; White, B.; Yaegashi, H.; Shimizu, M.; Yoshida, K.; Uemura, A.; Oikawa, K.; Abe, A.; et al. Genome sequencing of the staple food crop white Guinea yam enables the development of a molecular marker for sex determination. *BMC Biol.* **2017**, *15*.
7. Girma, G.; Natsume, S.; Carluccio, A.V.; Takagi, H.; Matsumura, H.; Uemura, A.; Muranaka, S.; Takagi, H.; Stavolone, L.; Gedil, M.; et al. Identification of candidate flowering and sex genes in white Guinea yam (D. rotundata Poir.) by SuperSAGE transcriptome profiling. *PLoS One* **2019**, *14*, e0216912.
8. Hamadina, E.I.; Craufurd, P.Q.; Asiedu, R. Flowering intensity in white yam (Dioscorea rotundata). *J. Agric. Sci.* **2009**, *147*, 469–477.
9. Lebot, V.; Abraham, K.; Kaoh, J.; Rogers, C.; Molisalé, T. Development of anthracnose resistant hybrids of the Greater Yam (Dioscorea alata L.) and interspecific hybrids with D. nummularia Lam. *Genet. Resour. Crop Evol.* **2019**, *66*, 871–883.
10. Simmonds, N.W.; Smartt, J. *Evolution of crop plants*; 2nd ed.; Harlow : Longman Scientific & Technical, 1995; ISBN 0582086434.
11. Asiedu, R.; Sartie, A. Crops that feed the World 1. Yams. *Food Secur.* **2010**, *2*, 305–315.
12. Girma, G.; Hyma, K.E.; Asiedu, R.; Mitchell, S.E.; Gedil, M.; Spillane, C. Next-generation sequencing based genotyping, cytometry and phenotyping for understanding diversity and evolution of guinea yams. *Theor. Appl. Genet.* **2014**, *127*, 1783–1794.
13. Heikrujam, M.; Sharma, K.; Prasad, M.; Agrawal, V. Review on different mechanisms of sex determination and sex- linked molecular markers in dioecious crops- A current update. *Euphytica* **2014**, *Accepted*.
14. Inoti, S.K.; Thagana, W.M.; Dodson, R. Sex determination of young nursery Jojoba (Simmondsia chinensis L .) plants using morphological traits in semi arid areas of Voi, Kenya. *J. Biol. Agric. Healthc.* **2015**, *5*, 113–124.
15. Harkess, A.; Leebens-Mack, J. A Century of Sex Determination in Flowering Plants. *J. Hered.* **2016**, *108*, 69–77.
16. Eagles, H.A.; Bariana, H.S.; Ogbonnaya, F.C.; Rebetzke, G.J.; Hollamby, G.J.; Henry, R.J.; Henschke, P.H.; Carter, M. Implementation of markers in Australian wheat breeding. *Aust. J. Agric. Res.* **2001**, *52*, 1349–1356.
17. Truţă, E.; Gille, E.; Tóth, E.; Maniu, M. Biochemical differences in Cannabis sativa L. depending on sexual phenotype. *J. Appl. Genet.* **2002**, *43*, 451–462.
18. Grewal, A.; Goyat, S. Marker assisted sex differentiation in dioecious plants. *J. Pharm. Res.* **2015**, *9*, 531–549.
19. Cormier, F.; Lawac, F.; Maledon, E.; Gravillon, M.C.; Nudol, E.; Mournet, P.; Vignes, H.; Chaïr, H.; Arnau, G. A reference high-density genetic map of greater yam (Dioscorea alata L.). *Theor. Appl. Genet.* **2019**, *132*, 1733–1744.
20. Thatte, K.S.; Deodhar, M.A. Study of Flowering Behavior and Sex Determination in Garcinia indica (Thomas-Du Pettite) Choisy by Means of Molecular Markers. *Biotechnology* **2012**, *12*, 232–237.
21. Bhattacharjee, R.; Ferguson, M.; Gedil, M.; Dumet, D.; Ingelbrecht, I. Field collection, preservation and large scale DNA extraction procedures for cassava (Manihot esculenta Crantz.). *African J. Biotechnol.* **2009**, *8*, 3424–3430.
22. Guo, Y.; Yang, G.Q.; Chen, Y.; Li, D.; Guo, Z. A comparison of different methods for preserving plant molecular materials and the effect of degraded DNA on ddRAD sequencing. *Plant Divers.* **2018**, *40*, 106–116.

23. Doyle, J.J.; Dickson, E.E. PRESERVATION OF PLANT SAMPLES FOR DNA RESTRICTION ENDONUCLEASE ANALYSIS. *Taxon* **1987**, *36*, 715–722.
24. Pereira, J.C.; Chaves, R.; Bastos, E.; Leitão, A.; Guedes-Pinto, H. An Efficient Method for Genomic DNA Extraction from Different Molluscs Species. *Int. J. Mol. Sci.* **2011**, *12*, 8086–8095.
25. Wilfinger, W.W.; Mackey, K.; Chomczynski, P. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *Biotechniques* **1997**, *22*, 474–481.
26. Bressan, E.A.; Rossi, M.L.; Gerald, L.T.S.; Figueira, A. Extraction of high-quality DNA from ethanol-preserved tropical plant tissues. *BMC Res. Notes* **2014**, *7*, 268.
27. Matasyoh, L.G.; Wachira, F.N.; Kinyua, M.G.; Thairu Muigai, A.W.; Mukiama, T.K. Leaf storage conditions and genomic DNA isolation efficiency in Ocimum gratissimum L. from Kenya. *African J. Biotechnol.* **2008**, *7*, 557–564.
28. Xin, Z.; Chen, J. A high throughput DNA extraction method with high yield and quality. *Plant Methods* **2012**, *8*, 1–7.
29. Till, B.J.; Jankowicz-Cieslak, J.; Huynh, O.A.; Beshir, M.M.; Laport, R.G.; Hofinger, B.J. Sample Collection and Storage. In Low-Cost Methods for Molecular Characterization of Mutant Plants: Tissue Desiccation, DNA Extraction and Mutation Discovery: Protocols; Springer International Publishing: Cham, 2015; pp. 9–11 ISBN 978-3-319-16259-1.
30. Liu, T.Y.; Iavarone, A.T.; Doudna, J.A. RNA and DNA targeting by a reconstituted Thermus thermophiles Type III-A CRISPR-Cas system. *PLoS One* **2017**, *12*, 1–20.
31. Zoundjihekpon, J.; Dansi, A. Biologie de la reproduction des ignames africaines. In *L'igname, Plant Seculaire et Culture d'Avenir*; Berthaud, J., Bricas, N., Marchand, J.-L., Eds.; 1998; pp. 231–240.
32. Orkwor, G..; Robert, A.; Ekanayake, I.. *Food Yams: Advances in Research*; Ibadan, Nigeria, 1998;
33. Nwachukwu, E.C.; Igbokwe, M.C. Proceedings of the 36th Annual Conference of Agriculturl Society of Nigeria.; Federal University of Technology: Owerri, Nigeria, 2002; pp. 16–17.
34. Doyle, J.J. Isolation of plant DNA from fresh tissue. *Focus (Madison).* **1990**, *12*, 13–15.