



Isolation and characterization of microsatellites for the yam *Dioscorea cayenensis* (Dioscoreaceae) and cross-amplification in *D. rotundata*

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ABSTRACT. *Dioscorea cayenensis* and *Dioscorea rotundata* are among the most important yam species for the humid and sub-humid tropics. We isolated nine polymorphic microsatellite markers using a microsatellite-enriched genomic library technique. The nine primer pairs were validated in 22 *D. cayenensis* accessions, and were tested for transferability in 26 *D. rotundata* accessions. The number of bands ranged from 2 to 4, with a mean of 3.11. *D. cayenensis* gave primer polymorphism information content values ranging from 0.37 to 0.62, while for *D. rotundata* the values ranged from 0.15 to 0.66. The *D* parameter in *D. cayenensis* ranged from 0.14 to 0.40, while in *D. rotundata* it ranged from 0.05 to 0.34. These SSR markers will be useful to characterize genetic diversity in *D. cayenensis* and *D. rotundata* accessions.

Key words: Molecular markers; SSR; Transferability; Tuber crop; Yams

INTRODUCTION

Yams (*Dioscorea* spp) represent a staple food for about 100 million people worldwide, where it is extensively cultivated and consumed in the humid and sub-humid tropics of several countries in Africa, Asia, and Latin America (FAO, 2011). *D. cayenensis* and *D. rotundata* (known as Guinea yams) are the most popular and economically important yams in West and Central Africa (Obidiegwu et al., 2009). In Brazil, these species, also called yellow yams (*D. cayenensis*) or white yams (*D. rotundata*), are mainly grown in the Northeast, producing few large tubers per plant (Santos, 2002). The taxonomy of these species is still controversial. Some authors consider them as part of a complex called *D. cayenensis/D. rotundata* (Dansi et al., 1999; Mignouna et al., 2002), while others consider them as two distinct species (Ramser et al., 1997; Mignouna et al., 2005).

These species are considered polyploid by most authors (Obidiegwu et al., 2009), although *D. rotundata* has also been considered diploid by Scarcelli et al. (2006). They are dioecious and perennial and are characterized by the presence of herbaceous stems with thorns, climbing habit and edible tubers rich in carbohydrates, vitamins and minerals (Lebot, 2009). These species are widely cultivated and consumed by communities that practice traditional farming (Siqueira, 2011). Thus, these communities play a key role in maintaining the genetic diversity of yams, as well as several other species of roots and tubers. However, the socioeconomic pressures suffered by farmers in these communities, accelerate the decline and loss of genetic variability in this crop (MMA, 2004).

In this context, there is a need to characterize the genetic variability of yam species maintained by traditional farmers, to estimate their level of diversity as well as to develop strategies for the conservation and maintenance of this diversity.

Several tools are available to estimate and characterize the genetic diversity, among which microsatellites or simple sequence repeats (SSR) have gained prominence due to their being codominant markers, multi-allelic and amplified via PCR, which facilitates their evaluation even with small amounts of DNA (Oliveira et al., 2006). Once developed, primers that amplify these regions can be easily used to characterize the genetic variability of any kind (Agarwal et al., 2008).

In this study, we report the isolation and characterization of nine microsatellite primers for the species *D. cayenensis* and the analysis of its cross-amplifications in *D. rotundata*.

MATERIAL AND METHODS

Genomic DNA was extracted from fresh young leaves of 22 accessions of *D. cayenensis*, originating from the municipalities of Iguape, Cananéia, Iporanga, Eldorado, Ubatuba, and Ilha Comprida in São Paulo State, and the municipalities of Joinville and Itajaí in Santa Catarina State. We also used the primer pairs obtained to evaluate 24 accessions of *D. rotundata* originating from Ubatuba and Mogi Guaçu, in São Paulo State, from Alhandra, Rio Tinto, Marcação, Sapé, Caaporã, Santa Rita, and Sobrado in Paraíba State, and the municipalities of São Joaquim do Monte, Bonito, Goiana, Aliança, Ferreiro, Recife, in Pernambuco State. DNA was extracted using the CTAB method (Doyle and Doyle, 1990).

To select fragments containing microsatellite markers, two genomic libraries were

constructed from genomic DNA of the accession DGC 4.0 (from Iguape-SP) following the protocol described by Billotte et al. (1999) with modifications.

Total DNA was digested using the enzyme *Afa* (Invitrogen cat. 15424-013, Carlsbad, CA, USA). The digestion product was ligated to adapters *Afa21* (5'CTCTTgCTTACgCgTggAC TA 3') (IDT, Integrated DNA Technologies, Inc. Cat. 15,350,710) and *Afa25* (5'pTAgTCCAC gCgTAAgCAAgAgCACA 3').

After binding, we performed a pre-amplification step using 3 µL ligation product, 20 µM primer *Afa21*, 5 µL 10X buffer, 2.0 mM MgCl₂, 200 mM dNTPs and 3 U Taq polymerase. PCR reactions were performed in a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA), in which the samples were initially submitted to 95°C for 4 min and then 20 cycles at 94°C for 30 s, 60°C for 1 min and finally 72°C for 2 min. After 20 cycles, the samples were kept for 8 min at 72°C. The PCR products were subjected to electrophoresis on a 1.0% agarose gel (w/v) in 1X TBE buffer containing Syber Safe, at 110 V for 1 h. The Gene Ruler 100 bp DNA Ladder Plus (Fermentas Life Sciences, Vilnius, Lithuania) was used as molecular weight marker.

The fragments were selected using magnetic beads (Promega, Cat. Z5481, Madison, WI, USA) and biotinylated probes containing tandem repeats of dinucleotides: biotIII (CTT)₁₀, biotIII (TA)₁₀, and III blot (GT)₁₀. The microsatellite-rich fragments were amplified in the same thermal cycler as above, at 95°C for 1 min and then 25 cycles of amplification. In each cycle, the samples were submitted to 94°C for 40 s, 60°C for 1 min and finally to 72°C for 2 min. After 25 cycles, the samples were kept for 5 min at 72°C. The PCR products were subjected to electrophoresis on a 1.0% agarose gel, as described above.

The amplified fragments were then ligated into a pGEM-T (pGEM-T Vector System I, Promega cat. A3600) cloning vector using *Escherichia coli* competent cells. The next step was a PCR to check whether the transformed clones had the inserts. After amplification, the fragments were separated on a 1.5% agarose gel. The transformed clones were stored at -80°C. After the selection of positive clones, the microsatellite fragments were extracted from the plasmids and sequenced.

The sequencing reaction was prepared in autoclaved ultrapure water with 4 µL plasmid, 0.5 mM primer SP6, 2 µL Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA) and 2 µL Save Money buffer (prepared with 10% (v/v) MgCl₂, 50 mM and 20% (v/v) Tris-HCl, pH 9.0, in autoclaved ultrapure water). Sequencing was performed at the Citrus Center of the Agronomic Institute in Cordeirópolis, São Paulo, Brazil. We used the DNA Analyzer 3730 sequencer (Applied Biosystems), and the sequences obtained were processed by the 3730/3730xl Data Collection Software v3.0 (Applied Biosystems).

The Websat program (available at <http://wsmartins.net/websat/>) was used to identify the microsatellite sequences obtained. The Primer 3 program (available at <http://bioinfo.ut.ee/primer3/>) was used to design primer pairs flanking the microsatellite. The parameters were set for obtaining a final amplification product in the range of 150 to 250 bp, GC percentage in the range of at least 50% and maximum 60%, the difference in annealing temperature of the primers varying between 55° and 70°C, and the difference in annealing temperature between primer pairs varying by 3°C at most.

The software Gene Runner v.3.1 was used to evaluate the quality of each primer pair obtained by selecting the most suitable for PCR. The software Chromas 2 was used to evaluate the quality of primer sequencing and the regions of the microsatellite. Allele sizes were estimated using the software GeneMarker v.1.95 (Softgenetics, State College, PA, USA).

The 22 accessions of *D. cayenensis* and 26 accessions of *D. rotundata* were analyzed

in a transferability test, using a “touchdown” cycling PCR program, using a “touchdown” cycling PCR program (Table 1). Electrophoresis was done in 7% denaturing polyacrylamide gels pre-run at 45 V/cm for 40 min, using 6- μ L samples previously denatured with 2 μ L loading buffer (95% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue, and 12.5% sucrose) at 95°C for five minutes. Electrophoresis was performed at a constant voltage of 45 V/cm. Gels were stained using a silver staining procedure (Creste et al., 2001) and photo-documented with a digital camera.

The number of bands was estimated visually and the polymorphism information content (PIC) was calculated according to Botstein et al. (1980) to estimate marker informativeness. The discrimination power (D) parameter (Tessier et al., 1999) was estimated for each primer according to the formula: $D_j = 1 - C_j = 1 - \sum_{i=1}^l p_i \frac{(Np_i - 1)}{N - 1}$, where D is the probability that two randomly selected individuals have a different and distinct banding pattern, C is the probability that two randomly selected individuals have a similar band pattern, and N is the number of individuals analyzed.

RESULTS AND DISCUSSION

Of the 12 designed primers, nine were successfully amplified and used to characterize the accessions of *D. cayenensis* and to test for cross-amplification in *D. rotundata*. The three remaining primer pairs were excluded due their being monomorphic or nonspecific.

The SSR primers obtained in this study were used as dominant markers to evaluate the genetic divergence between germplasm accessions of *D. cayenensis* and *D. rotundata*. A total of 28 bands were obtained for the nine primers. The number of bands ranged from 2 to 4, with an average of 3.11 bands per primer pair (Table 1). The PIC values of the nine microsatellite primers in *D. cayenensis* ranged from 0.37 to 0.62, with an average of 0.54. The highest PIC was found for the Dca11 primer, with a value of 0.62, containing the highest band number (4 bands), also obtained with Dca5 primer. The PIC values in *D. rotundata* ranged from 0.15 to 0.66, with an average of 0.43. The highest PIC was also found for the Dca11 primer, at 0.66. Obidiegwu et al. (2009) assessed the genetic diversity of 219 accessions of *D. cayenensis/D. rotundata* from Benin, Congo, Côte d’Ivoire, Equatorial Guinea, Gabon, Ghana, Nigeria, Sierra Leone, and Togo on the basis of 15 microsatellite primers, and observed that PIC ranged from 0.37 to 0.80, with an average of 0.65.

We compared the markers for efficiency in genotype identification by determining the discrimination power (D) parameter for each primer (Tessier et al., 1999). The D parameter in *D. cayenensis* ranged from 0.14 to 0.40, with an average of 0.23. Higher D values were found for the Dca11 (0.40) and Dca10 (0.36) primers (Table 1). The D parameter in *D. rotundata* ranged from 0.05 to 0.34, with an average of 0.21. The highest D value was found for primer Dca11 (0.34) (Table 1). Siqueira et al. (2011) developed nine primers specific for *D. alata*, observing D values ranging from 0.15 to 0.91, with an average of 0.68. Thus, the primers developed for *D. cayenensis/D. rotundata* showed low D values, indicating low power in discriminating the accessions used in this study. However, these primers may perform well in other sets of accessions, thereby assisting in studies of the genetic diversity of yam.

The results of the cross-amplification tests showed that all primers allowed transferability to the species *D. rotundata*, indicating that the nine microsatellite primers reported in this study are a potentially useful tool to evaluate genetic variation and population structure in these two species.

Table 1. Characterization of nine microsatellite primers in *Dioscorea cayenensis* and cross-amplification in *Dioscorea rotundata*.

Primer	Primer sequence (5'-3')	Repeat	Size range (bp)	T _a (°C) Touchdown	GeneBank	N° of bands	PIC		Parameter D		Transferability
							<i>D. cayenensis</i>	<i>D. rotundata</i>	<i>D. cayenensis</i>	<i>D. rotundata</i>	
Dea2	F: TGGATTGTGAAGCCATTGTT R: GCATGACCTTCAAAAATACACC	(GT) ₅	187-193	60°-50°C	JX282392	3	0.56	0.51	0.30	0.18	+
Dea3	F: CAGCCACTTGCATAAATG R: AGCTTGTAGTCGGTGAAGG	(GT) ₁₀	202-220	55°-45°C	JX282393	3	0.55	0.42	0.16	0.11	+
Dea4	F: ATGGGGCACATAAATTAGGG R: CAATAAATAAACACTTGAGACACGA	(CA) ₈ +(TA) ₆	276-290	55°-45°C	JX282394	3	0.46	0.60	0.18	0.27	+
Dea5	F: AATGTTTGTATCCCTCAATGG R: CACCCGTAATTGGTTACATGG	(AC) ₁₆	198-250	55°-45°C	JX282395	4	0.59	0.19	0.32	0.28	+
Dea6	F: TGCTTACTCTTGACCCCTAA R: TGCATCCCAACCCTTGATTA	(TG) ₈	190-210	55°-45°C	JX282396	3	0.59	0.40	0.32	0.17	+
Dea8	F: TACGAGGCTTAGCCGTGAT R: CGTATCCCTTGTAGTAAAGCAA	(CT) ₁₈	197-205	55°-45°C	JX282398	2	0.37	0.15	0.14	0.05	+
Dea9	F: GACAAATCAATGAGGCCAAC R: TGGCTTAACTATGGCGGACT	(TC) ₃	195-205	55°-45°C	JX282399	3	0.50	0.40	0.18	0.17	+
Dea10	F: CGGGTGTGATGTATGAGAGC R: GCTGGTTCCTTAAATGATGG	(GA) ₁₈	136-195	60°-50°C	JX282400	3	0.59	0.53	0.36	0.28	+
Dea11	F: CCCATGAAATGAGGAACCCCTA R: GCCCAGCAAATGGTAGATGT	(AG) ₁₄	73-83	60°-50°C	JX282401	4	0.62	0.66	0.40	0.34	+
Average						3.11	0.54	0.43	0.23	0.21	

T_a = annealing temperature of the primer pairs; PIC = polymorphism information content; D = discrimination power. (+) = successful amplification with one or more bands of a size similar to that of the original sequenced clone; (-) = no amplification.

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