RESEARCH

Development of Genomic Simple Sequence Repeat Markers for Yam

Muluneh Tamiru,* Shinsuke Yamanaka, Chikako Mitsuoka, Pachakkil Babil, Hiroko Takagi, Antonio Lopez-Montes, Alieu Sartie, Robert Asiedu, and Ryohei Terauchi

ABSTRACT

Yam (*Dioscorea* spp.) is a major staple crop widely cultivated for its starchy tubers. To date, very few marker resources are publicly available as tools for genetic and genomic studies of this economically important crop. In this study, 90 simple sequence repeat (SSR) markers were developed from an enriched genomic library of yellow Guinea yam (*D. cayenensis* Lam.). Cross-amplification revealed that 85 (94.4%) and 51 (56.7%) of these SSRs could be successfully transferred to the two major cultivated species of *D. rotundata* Poir. and *D. alata* L., respectively. Polymorphisms in 30 markers selected on the basis of reliability and reproducibility of DNA bands were evaluated using a panel of 12 *D. cayenensis*, 48 *D. rotundata*, and 48 *D. alata* accessions. Accordingly, number of alleles per locus ranged from 2 to 8 in *D. cayenensis* (mean = 3.9), 3 to 30 in *D. rotundata* (mean = 13.9), and 2 to 22 in *D. alata* (mean = 12.1). The average observed and expected heterozygosities were 0.156 and 0.634 (*D. cayenensis*), 0.326 and 0.853 (*D. rotundata*), and 0.247 and 0.836 (*D. alata*), respectively. Clustering based on six SSRs that were polymorphic in at least four of the five cultivated *Dioscorea* species studied, including *D. cayenensis*, *D. rotundata*, *D. alata*, *D. dumetorum* (Kunth) Pax., and *D. bulbifera* L., detected groups consistent with the phylogenetic relationships of the species except for *D. dumetorum*. These new SSR markers are invaluable resources for applications such as genetic diversity analysis and marker-assisted breeding.

M. Tamiru, C. Mitsuoka, and R. Terauchi, Iwate Biotechnology Res. Center, Narita 22-174-4, Kitakami, Iwate 024-0003, Japan; S. Yamanaka, P. Babil, and H. Takagi, Japan International Res. Center for Agricultural Sciences, 1-1 Ohwashi, Tsukuba, Ibaraki 305-8686, Japan; A. Lopez-Montes, A. Sartie, and R. Asiedu, International Institute of Tropical Agriculture, PMB 5320, Ibadan, Nigeria; A. Sartie (current address), AgResearch Ltd., Grassland Res. Center, Private Bag 11008, Palmerston North 4442, New Zealand. Muluneh Tamiru and Shinsuke Yamanaka contributed equally to this work. Received 23 Oct. 2014. Accepted 22 Feb. 2015. *Corresponding author (olimt@ibrc.or.jp).

Abbreviations: *A*, number of alleles; CTAB, cetyl trimethyl ammonium bromide; *H*e, expected heterozygosity; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; *H*o, observed heterozygosity; IITA, International Institute of Tropical Agriculture; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; SSR, simple sequence repeats; YM, yam markers.

Yam (*Dioscorea* spp., Dioscoreaceae) is a multispecies crop widely distributed in Africa, Asia, Oceania, and South America. The genus *Dioscorea* L. comprises about 450 species (Wilkin et al., 2005), but only 10 species have significant importance as food (Lebot, 2009). Of the cultivated species, Guinea yams (*D. rotundata* Poir. and *D. cayenensis* Lam.) are extensively grown for human consumption in the yam belt of West and Central Africa, a region where yam is a crop of great economic and cultural importance for millions of people. Overall, Africa accounts for 96% of the global yam production (FAOSTAT, 2013). Yam production has increased steadily over the last decades, mainly through planting of more land with traditional cultivars (Mignouna et al., 2008). Yam breeding programs have so far focused on developing improved varieties through

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selection of superior landraces as well as crossing of genotypes selected for desirable traits such as yield, tuber quality, agroecological adaptation, and resistance to biotic and abiotic stresses (Asiedu et al., 1998). However, the use of advanced breeding methods in this crop has remained slow and difficult, mainly because very little is known about yam genetics.

Yam breeding is constrained by several inherent attributes of the crop including a long growth cycle, inconsistent or no flowering, dioecy, nonsynchronous flowering of parental genotypes, polyploidy, and high heterozygosity (Asiedu et al., 1998). Additionally, the available yam genetic resource is poorly characterized, limiting utility of the existing diversity in crop improvement programs. Efforts to generate genetic tools and genomic resources for accelerated yam breeding are currently underway including whole genome sequencing of *D. rotundata* and isolation of molecular markers such as microsatellites or simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs).

Molecular markers are important tools for applications such as estimating genetic diversity and phylogenetic relationships, cultivar identification, mapping of major-effect genes and QTLs, assessing population structure, selection of desirable genotypes in breeding programs, and for authentication of progenies obtained from genetic crosses. Simple sequence repeat markers are very popular because they are codominant and multiallelic and, thus, are more informative than dominant markers (Zalapa et al., 2012). To date, only a few genomic SSR markers have been developed for *D. cayenensis* and the other *Dioscorea* species (Terauchi and Konuma, 1994; Mignouna et al., 2003; Mizuki et al., 2005; Tostain et al., 2006; Hochu et al., 2006; Siqueira et al., 2011; Silva et al., 2014) (Table 1). In the present work, we describe the development of genomic SSR markers for yellow Guinea yam (*D. cayenensis*) using the method of enriched microsatellite libraries and demonstrate their use in multiple *Dioscorea* species.

MATERIALS AND METHODS

DNA Isolation and Plant Materials

DNA for constructing a genomic library was extracted from lyophilized leaf samples collected from a *D. cayenensis* accession TDc2082 grown at the International Institute of Tropical Agriculture (IITA) using the DNeasy plant mini kit (Qiagen). Additionally, a total of 133 yam accessions representing six *Dioscorea* species and consisting of 12 *D. cayenensis*, 48 *D. rotundata*, 48 greater or water yam (*D. alata* L.), 12 trifoliate or bitter yam (*D. dumetorum* (Kunth) Pax.), two lesser or Asiatic yam [*D. esculenta* (Lour.) Burkill], and 11 aerial yam (*D. bulbifera* L.) were selected from the IITA yam germplasm collection and used for testing the polymorphisms and transferability of the isolated SSR markers as well as for assessing the genetic relationship among the species. For these purposes, DNA was isolated by a standard CTAB (cetyl trimethyl ammonium bromide) method after an initial washing of samples with 0.1M HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer to remove contaminating polysaccharides.

Table 1. Genomic simple sequence repeat (SSR) markers so far developed for *Dioscorea* species.

Number of SSRs developed	Species used	Source
6	*D. tokoro*	Terauchi and Konuma, 1994
9	*D. rotundata*	Mignouna et al., 2003
10	*D. japonica*	Mizuki et al., 2005
16	*D. alata, D. abyssinica,* and *D. praehensilis*	Tostain et al., 2006
8	*D. trifida*	Hochu et al., 2006
9	*D. alata*	Siqueira et al., 2011
9	*D. cayenensis*	Silva et al., 2014

Construction of Genomic Libraries and Cloning

Microsatellites enrichment was undertaken following the method described by Glenn and Schable (2005). Briefly, DNA was first digested with restriction enzymes *Rsa*I and *Bst*UI (New England Biolabs) in separate reactions, and fragments generated by *Rsa*I were ligated to double-stranded SuperSNX linkers (SuperSNX24 Forward 5′-GTTTAAGGCCTAGCTAGCAGCAGAATC and SuperSNX24+4P Reverse 5′-GATTCTGCTAGCTAGGCCTTAAACAAAA). Linker-ligated DNA was enriched with three mixes of biotinylated microsatellite oligo [Mix 2 = $(AG)_{12}$, $(TG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(ATC)_8$; Mix 3 = $(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$, $(AATG)_6$, $(ACAG)_6$, $(ACCT)_6$, $(ACTC)_6$, $(ACTG)_6$; Mix 4 = $(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, $(AGAT)_8$], which were then captured on magnetic streptavidin beads (Dynal). Unhybridized DNA was removed by washing and the remaining DNA was eluted from the beads, amplified by polymerase chain reactions (PCR) using the forward SuperSNX24 primer, and cloned with Invitrogen TOPO-TA Cloning Kit (Invitrogen; http://www.lifetechnologies.com, accessed 15 May 2015).

Simple Sequence Repeat Identification and Primer Design

Following PCR amplification of colonies, plasmids were extracted from colonies containing inserts of the expected sizes and were sequenced with the M13 forward and reverse primers using the BigDye DNA sequencing kit (Applied Biosystems). Sequences from both strands were assembled and edited using GENETYX for Mac (GENETYX-MAC; https://www.genetyx.co.jp, accessed 15 May 2015). Microsatellites were identified using MsatCommander version 1.8.1 (Faircloth, 2008), and primers were designed with the Primer3 software provided in the GENEYX-MAC program.

Polymerase Chain Reaction and Electrophoresis

All PCRs were performed on a DNA Engine PTC-200 thermal cycler (Bio-RAD) in a total volume of 10 μL containing 1X ExTaq Buffer (TaKaRa), 200 μM of dNTPs, 1 μM of each forward and reverse primer, 0.5 U of Takara ExTaq HS, and approximately 20 ng of template DNA. The amplification condition was 3 min initial denaturation at 94°C, followed by 35

Table 2. List of simple sequence repeat (SSR) markers developed for yellow yam (*Dioscorea cayenensis*) with their corresponding repeat motif, sequence information, melting temperature (Tm), guanine-cytosine (GC) content, expected product size, and transferability to *D. rotundata* and *D. alata*.

No.	Locus	Repeat motif	Primers (5´ to 3´)	Tm	GC	Product Size	Cross-species amplification *D. cayenensis*	*D. rotundata*	*D. alata*
				°C	%	bp			
1	YM01	$(AC)_8$	F: GTGTGTGGGATTTTGTCAATC	58	43	404	+	+	–
			R: AGGTTTACACACATCCCCTTT	57	43				
2	YM02	$(AAG)_6$	F: TAGATTTCGCTTTTCCACTAGC	58	41	263	+	+	+
			R: CCTAATCATCATCATCGTCATC	57	41				
3	YM03	$(GAT)_6$	F: TCACTCAAACAATGAGCGTAG	57	43	202	+	+	+
			R: GATGGCTGCTGCATGACTG	60	58				
4	YM04	$(AC)_{21}$	F: AGTTCATCACAACTCATCCTCA	57	41	320	+	+	–
			R: CCTAGCAAGCATGTCAATCTAC	57	45				
5	YM05	$(AAG)_8$	F: AGGATTATCACTGAAAGGGCT	57	43	140	+	+	+
			R: CCTTCCAATTACTCTCCAAGA	56	43				
6	YM06	$(AAG)_{18}$	F: ACAGAGCTGTTGACACAAACA	57	43	398	+	+	–
			R: CCTCAATGAACCTTTGGTCTA	57	43				
7	YM07	$(CTT)_{15}$	F: AGCATTGGGTCCTTTCATCC	59	50	203	+	+	–
			R: ACAATTCACACAAAGCATGGC	59	43				
8	YM08	$(AG)_{24}$	F: TCTTAGGCTTTGGGCAGGG	60	58	166	+	+	–
			R: AGTATGCCTACCCTGTTCTTC	58	48				
9	YM09	$(CTT)_{12}$	F: AGGAACATTCCCACTCAGTTATG	59	43	193	+	+	+
			R: ATTGGGCAAGTGTGGTGTG	59	53				
10	YM10	$(GAT)_7$	F: ACCCAAAATATTCTCCCCATTATAC	57	36	348	+	+	–
			R: TTGACACTCATCTTATATTGCTCC	57	38				
11	YM11	$(AG)_{17}$	F: GGATGGCGTAGAGGAAGAGG	60	60	205	+	+	–
			R: GGATAAGACCACGAGTGTTGC	60	52				
12	YM12	$(ATC)_5…(AAC)_8$	F: TGAGCATTCTTGTTTTGCCG	58	45	215	+	+	+
			R: CTTTCAGGGCGTGCATGG	60	61				
13	YM13	$(CTT)_8$	F: CCAATCACATCACGTCTAGTCT	57	45	328	+	+	+
			R: GACAATAGAAACTTCGAGACCC	57	45				
14	YM14	$(GAT)_{10}$	F: TGACTTGAGTAGATCAGGTTGTC	58	43	196	+	–	–
			R: AAGTTGAAGCTTTCCTATAGACG	57	39				
15	YM15	(CTT)7	F: CCATCTCCTCCCTTATCTACAC	57	50	485	+	+	+
			R: GGGATTGAAGTTCCAGAGACTA	57	45				
16	YM16	$(CT)_{13}$	F: TGAAGAGAATGTTGAGATCGTACC	59	42	150	+	+	–
			R: TATCCGGCCCTCTCATTGG	59	58				
17	YM17	$(AC)_8$	F: TCCCTCAATTAAAGCATAGCCTC	59	43	181	+	+	+
			R: AGCCACCAAACATCTTGCTC	60	50				

(cont'd.)

cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. Polymerase chain reaction products were electrophoresed on 8% polyacrylamide gel (mono:bis = 29:1) and in 0.5X TBE buffer at 150 V for 90 min. After taking photographs, gel images were analyzed with Dolphin-1D Gel analysis software ver. 2.7 (Wealtec Corp.).

RESULTS

Isolation of Simple Sequence Repeats from *D. cayenensis* Genomic DNA Library

A total of 432 positive clones harboring inserts of the expected sizes were identified by PCR and sequenced by Sanger sequencing. Of these, 171 generated SSR-containing unique sequences at least with 6-dinucleotide, 5-trinucleotide, or 4-tetranucleotide repeats as identified by MsatCommander (Faircloth, 2008), but only 110 contained adequate flanking regions for primer design. Primer pairs were designed for these 110 products, which were then tested for PCR amplification and reproducibility. Accordingly, 90 produced reliable PCR products of the expected sizes. These SSR-containing sequences were named as YM (yam markers) followed by consecutive numbers (Table 2).

Polymorphisms and Transferability to Other *Dioscorea* Species

Cross-species amplification of the 90 *D. cayenensis* markers was evaluated in two other cultivated *Dioscorea* species: the major African species of *D. rotundata* and the widely distributed Asian species of *D. alata*. Eighty-five (94.4%)

Table 2. Continued.

No.	Locus	Repeat motif	Primers (5´ to 3´)	Tm	GC	Product Size	Cross-species amplification D. cayenensis	D. rotundata	D. alata
				°C	%	bp			
18	YM18	$(GT)_{19}$	F: GACATTGGGGATCTCTTATCAT	57	41	266	+	+	+
			R: TAGCAGCAGTAACGTTAAGGAA	57	41				
19	YM19	$(CT)_{18}$	F: ACGGAAGCAGCAAGAGGAG	60	58	219	+	+	–
			R: GTGTCATCAGCATCTGGGC	59	58				
20	YM20	$(CT)_{12}$	F: GTTGCCACACTTGGTGCC	60	61	249	+	–	–
			R: TGGTGAGACCTGAGAATAATTAATGG	60	38				
21	YM21	$(GAT)_{5}$	F: AATGATGCATCTGAGGATAGTG	57	41	340	+	+	+
			R: GATGCTATTACGACAACCTTGA	57	41				
22	YM22	$(GTT)_{6}$	F: CGACTAGATTTTCTTGTTGGTG	57	41	282	+	+	–
			R: GGTCACTTTGTTCTAATGCAAG	57	41				
23	YM23	$(AG)_{14}$	F: TTAAGACTTGCAGGGTTAAAGG	58	41	200	+	+	+
			R: GTGGCTAGTTTTTGTAGCTGGT	58	45				
24	YM24	$(GTT)_{11}$	F: GGTGTTGTTGGGTTTCATTGTC	59	45	188	+	+	+
			R: TCCCTCTTCTCATTTCACTCCC	60	50				
25	YM25	$(AG)_{30}$	F: GATGGAGATGAGGAGGCCG	60	63	237	+	+	–
			R: TTCGAAGCCAGAGCAAGTG	59	53				
26	YM26	$(AG)_{22}$	F: CACTAGCTCCGAAGAAGAGAG	58	52	250	+	+	–
			R: AGGAGTGTTGGTGCTCATATC	58	48				
27	YM27	$(GTT)_{8}$	F: TCCAGCTCTTTAGCACAGG	58	53	231	+	+	+
			R: AGGAGCATAGGCAACAAGC	59	53				
28	YM28	$(CTT)_{8}\ldots(CTT)_{14}$	F: CCATTCCTATTTAAGTTCCCCT	58	41	333	+	+	–
			R: GATGAAGAAGAAGGTGATGATG	56	41				
29	YM29	$(AAC)_{18}\ldots(AG)_{13}$	F: AAGGGCACCCTACATAATAAGA	57	41	352	+	+	–
			R: GAGATCTTGGAGATCATCACTG	56	45				
30	YM30	$(GT)_{16}$	F: CCACAACTAAAAACACATGGAC	57	41	212	+	+	+
			R: GTGGTAGGGTGTGTAGCTTCTT	57	50				
31	YM31	$(AAG)_{9}$	F: AAGCCTAGTCGATGGGTGG	60	58	221	+	+	–
			R: TGCTGTTCCAACTTCCAAGC	60	50				
32	YM32	$(CT)_{24}$	F: GAGGTCTGCGACGGATTTG	59	58	244	+	+	+
			R: TCGCATTCTTCATCCTCTTCAC	59	45				
33	YM33	$(AAG)_{13}$	F: ACCATGGGATGAAGGGAAGG	60	55	199	+	+	+
			R: GCATATGGTGCATGGGAGC	60	58				
34	YM34	$(AG)_{16}$	F: GGTAATAGAGGGCAAAGTGGC	59	52	215	+	+	–
			R: AGACCTCCTACCATGCTCAAG	60	52				
35	YM35	$(GT)_{8}$	F: GCTCTAGCAAACAATCCAATC	57	43	271	+	+	–
			R: CCCTATACGCATGAAAGTAACA	57	41				
36	YM36	$(GAT)_{5}$	F: CCTTACCACCGGACTCCTC	60	63	156	+	+	+
			R: TGCAGCAATACACCGGAAC	59	53				
37	YM37	$(CT)_{15}$	F: TCTCTTCTTCTTTGGCATCGC	59	48	216	+	+	+
			R: GGCAGCGAGTTCCTCAAATC	60	55				
38	YM38	$(GT)_{8}$	F: GAACCCCTGTCAAGTGAGC	59	58	152	+	+	+
			R: CTGAAGCTCGAACAAATGCAG	59	48				
39	YM39	$(GT)_{7}$	F: TGGAGAGAGTCAAACACTAGAGG	60	48	225	+	+	–
			R: GGGCCACTCAGGAAAGAAC	59	58				
40	YM40	$(AG)_{11}$	F: ACCCTAGCCATCTCTTCACC	59	55	157	+	+	–
			R: CTGACACCACCCGCAAAAG	60	58				
41	YM41	$(AAG)_{8}$	F: GCCAATTCTTACAACTGTGATGG	59	43	245	+	+	–
			R: TGGCAAACTATAAATCAGCCCAC	60	43				
42	YM42	$(CT)_{29}$	F: ACTCCAGGTGATTCTTGGC	58	53	214	+	+	–
			R: AGAGGCTGTAGTTGTCCCAG	60	55				
43	YM43	$(AAG)_{9}(GA)_{7}$	F: GCCTTGTTTTGTTGATGCTTCG	60	45	178	+	+	–
			R: CCAGCCCACTAATCCCTCC	60	63				

(cont'd.)

Table 2. Continued.

No.	Locus	Repeat motif	Primers (5′ to 3′)	Tm	GC	Product Size	Cross-species amplification D. cayenensis	D. rotundata	D. alata
				°C	%	bp			
44	YM44	$(AG)_{20}$	F: CGCAACCAGCAAAGGATTTA	61	45	156	+	+	+
			R: ATTCTGTCTCTCAAAACCCCT	57	43				
45	YM45	$(AT)_{6}…(AT)_{7}$	F: GCTGTTTTTGGGATTTAGTAGG	57	41	235	+	+	+
			R: GCAACTAGATATGCTTGGACAC	57	45				
46	YM46	$(AC)_{8}…(AC)_{15}$	F: ACTTGACATCCAGAAGGTGC	59	50	250	+	+	+
			R: GCTGTAACTTATCAAGGGTAGC	57	45				
47	YM47	$(AAG)_{8}…(AAG)_{6}$	F: AGAGGAAGAAGAGGTAGTCAAAG	58	43	153	+	–	–
			R: TCTCCAGTTCCCCAAAGAGC	60	55				
48	YM48	$(AC)_{6}…(CT)_{16}$	F: TTGTGGGATCTGGTTGGAAG	58	50	150	+	–	+
			R: AAAGACTGTGGCCTAGAAGTG	58	48				
49	YM49	$(AG)_{26}$	F: TGGGGTGAGAGAGTAAGTGG	59	55	163	+	+	+
			R: TCACCGGGGATCTTCTTGC	60	58				
50	YM50	$(CTT)_{9}$	F: TTGCCCTTGGGATGTAGGG	60	58	234	+	+	–
			R: CATCCCCGTTGTATCCTGC	59	58				
51	YM51	$(AG)_{6}…(AG)_{17}$	F: GAATACATATGGTGCATTCGAG	57	41	356	+	+	–
			R: GCTGCTTACAACTGACAAAGTC	57	45				
52	YM52	$(GT)_{19}…(AG)_{19}$	F: TGCATAGAGTGAGAGCTTAAGAG	56	43	384	+	+	+
			R: ATGGGTAGTTGAGCAAGAGAAT	57	41				
53	YM53	$(AG)_{24}$	F: CTCATAAGCAGAGCCTTCTCTC	58	50	322	+	+	+
			R: TACAGTCCCTGTTTGAGCATAG	57	45				
54	YM54	$(CT)_{11}…(AC)_{16}$	F: CACTTGCTCTCTCATCGGC	59	58	162	+	+	–
			R: TTGACAACCTCTATTTTGCCC	57	43				
55	YM55	$(CT)_{11}…(AC)_{18}$	F: TCTTCCGAGATATACACATCCA	57	41	380	+	+	+
			R: ACAGTGACAATGAGAAGGAACA	57	41				
56	YM56	$(CTT)_{8}$	F: CCTTTCTGCTTGCTTTTTGT	57	40	284	+	+	+
			R: GGTGTGATAAACTTCAACCTCA	57	41				
57	YM57	$(AAC)_{22}$	F: CGTGGTTGTTGGGTGTAGC	60	58	170	+	+	–
			R: CCTTGGCACCATTGCCTTG	60	58				
58	YM58	$(CTT)_{13}$	F: TCTGGGGCACTGTCCTTTC	60	58	199	+	+	+
			R: CCACATGGACTGGAATAGCTC	59	52				
59	YM59	$(AC)_{23}$	F: CCCGTGCACTTGTAGGAAG	59	58	249	+	+	–
			R: TCACAAGCAAATGAGGGAAAC	58	43				
60	YM60	$(AG)_{18}…(AG)_{7}$	F: AGAAACCCTGGTGTGTGGG	60	58	199	+	+	–
			R: CATGTCTGCATCTTGGGGC	60	58				
61	YM61	$(GTT)_{26}$	F: AGTGGTGCTGTAGTAACTGGAA	57	45	252	+	+	–
			R: CATGACTACCTTTCCTCAATCA	27	41				
62	YM62	$(CT)_{10}…(CT)_{25}$	F: GAGCTCTCCTCTAGACCTTCAC	60	55	195	+	+	+
			R: CCAACGGCCTAGATTGCTC	59	58				
63	YM63	$(AAG)_{12}…(AT)_{6}$	F: GACAATAGAAACTTCGAGACCC	57	45	426	+	+	–
			R: GATGACAATATGTCTTCATCGC	57	41				
64	YM64	$(AG)_{33}$	F: CCGTATTATATATGGGTGACCA	56	41	303	+	+	+
			R: CAAAGCAAACAAGGATGACA	57	40				
65	YM65	$(AG)_{21}$	F: ACAAATGCACGCTCTGAAGG	60	50	183	+	+	+
			R: GGGCAGTAGAATTTGGTGCG	60	55				
66	YM66	$(AT)_{7}(GT)_{17}$	F: ATATTGACTGACCACCAGATCA	57	41	246	+	+	+
			R: GAAGAGTCTTGGATTTCTACCA	55	41				
67	YM67	$(GT)_{22}$	F: GGCTGACTTGTGTAAACTCTTG	57	45	383	+	+	+
			R: TGTAGATGAGGCAAGAAGTGAT	57	41				
68	YM68	$(GT)_{15}$	F: TAGGAAGGCAGTCTCCCGC	62	63	208	+	+	+
			R: CCACGAAATACTGAACCCCG	59	55				
69	YM69	$(CT)_{6}…(AGTT)_{5}$	F: CTCTCTACCTCCCAACAAAAAC	57	45	229	+	+	+
			R: AATCTTGCACCACCTTTTCTAC	57	41				

(cont'd.)

Table 2. Continued.

No.	Locus	Repeat motif	Primers (5′ to 3′)	Tm	GC	Product Size	Cross-species amplification *D. cayenensis*	*D. rotundata*	*D. alata*
				°C	%	bp			
70	YM70	$(AC)_{17}$	F: GTGCTACAACTTTGATTTCC	52	40	318	+	+	+
			R: GATGCTGTGCAGTTACTGTTTT	57	41				
71	YM71	$(GT)_9$	F: TTGGAATCAAGCCAATGCTC	57	45	500	+	+	+
			R: ACCCAGCATCTATCTTATTTTGC	57	39				
72	YM72	$(AC)_{17}$	F: ATATGTGAGCATAGCAAGAGGA	57	41	169	+	+	–
			R: GTCACCTACCTATATAAACCCATGC	60	44				
73	YM73	$(AC)_{16}$	F: GACATCGTTCTTTCATATAGCG	57	41	326	+	+	+
			R: AAGTTATGGACCTCAGGACTGT	57	45				
74	YM74	$(CT)_{12}...(GT)_{19}$	F: TGGTGTTTGAGAATGGAGGATTG	60	43	480	+	+	+
			R: ACTTGATCTTTGTCTTGATGGC	58	41				
75	YM75	$(GT)_9...(GT)_8$	F: TCGCTCAACCTAATCCTCTATT	57	41	350	+	+	+
			R: TCAAACCAGCCAAAACATC	57	42				
76	YM76	$(AC)_9$	F: ACAGCTACCACCTCGAAAGTAT	57	45	328	+	+	+
			R: ATCAAGGAAGAGACATGGAAGT	57	41				
77	YM77	$(GT)_{15}$	F: ATGTGGCCCTTTCTCTTGC	59	53	242	+	+	+
			R: GAGTAGCGGTGGTTGTGTG	59	58				
78	YM78	$(GT)_{10}$	F: ATGACTACTGCAAGGACAACAG	57	45	310	+	+	+
			R: GGTGATATGCATGATTCAACCT	59	41				
79	YM79	$(GT)_{34}$	F: AGTGCAAGACCTTGGGTGC	61	58	220	+	–	–
			R: GCTCTTTCCACCCTCAATGC	60	55				
80	YM80	$(CTT)_{13}$	F: CCGCCCAATCACATCACATC	60	55	245	+	+	+
			R: TCCCAAGAAGTCTGAGCCG	60	58				
81	YM81	$(AG)_{21}$	F: TTTGTTGCCATCCCAAGCC	60	53	238	+	+	–
			R: GTTGGCATCACCACTAGTCC	59	55				
82	YM82	$(GT)_9$	F: TGAGTGGGACAATCATCACC	58	50	221	+	+	–
			R: TTTCGCATGACACGGCTTC	60	53				
83	YM83	$(GT)_{13}$	F: TCGGAATTCAACTGTGATGGC	60	48	239	+	+	+
			R: AGCACACCATTCACACATAGG	59	48				
84	YM84	$(GT)_9$	F: TGCAAAGATCTCCTTATATTTGGC	58	38	358	+	+	–
			R: TGTCATTTGGTGTAATAGTGTCAAC	59	36				
85	YM85	$(GT)_{22}...(AG)_{20}$	F: AATCAACAATTGAAGTGCAAAG	57	32	254	+	+	–
			R: GATGGGTTACACACTCTCACAC	57	50				
86	YM86	$(AC)_7...(AC)_{15}$	F: GCGCAAAGCTGTATCTGGC	61	58	176	+	+	+
			R: CTGTCCCATTAGGGCACTTG	59	55				
87	YM87	$(AC)_{13}$	F: AGGCCGAGAAAGAAATGATATTG	58	39	300	+	+	+
			R: GAGAAGGTTGGTAATTGCCCC	60	52				
88	YM88	$(AAT)_5...(ACAT)_9$	F: CTTTATCCATTAGAGCTTTGGG	57	41	257	+	+	+
			R: GTGAAATAACCTAGCAAATCCC	57	41				
89	YM89	$(ACT)_{54}$	F: CTGAGCTTAAGTAAGGTAGTTTGAG	58	40	250	+	+	+
			R: TGCATGCTCATACGTGAGTTC	59	48				
90	YM90	$(GT)_8$	F: GTGTGTGGATGGAGTTTCAAT	57	43	304	+	+	+
			R: GAATACCCCCAACAGATGTAAT	57	41				

SSRs successfully amplified PCR products of the correct size in *D. rotundata*, while 51 (56.7%) gave successful amplification in *D. alata* (Table 2). This higher cross-amplification of the new markers demonstrated their potential utility as molecular tools for various applications including improving the breeding efficiency of these economically important species of *Dioscorea*.

Polymorphisms in a set of 30 SSR markers selected on the basis of their reproducibility and reliability were further evaluated using 108 genotypes belonging to three *Dioscorea* species: *D. cayenensis* (12 accessions), *D. rotundata* (48 accessions), and *D. alata* (48 accessions) (Table 3). All the SSRs were polymorphic in *D. cayenensis*, with the number of alleles detected (A), as well as the observed (H_o) and expected (H_e) heterozygosities ranging from 2 to 8 (mean = 3.9), 0.00 to 1.00 (mean = 0.156), and 0.403 to 0.833 (mean = 0.634), respectively. The same markers were also polymorphic in *D. rotundata*, with A ranging from 3 to 30 (mean = 13.9), while H_o and H_e ranged from 0.00 to 0.938 (mean = 0.326) and 0.559 to 0.950

Table 3. Summary statistics for 30 *Dioscorea cayenensis* simple sequence repeat (SSR) markers used to assess the diversity in germplasm accessions of *D. cayenensis*, *D. rotundata*, and *D. alata*. *A,* number of alleles; *Ho,* observed heterozygosity; *He,* expected heterozygosity; *F,* fixation index; –, no amplification; *N*, number of individuals.

		D. cayenensis (*N* = 12)				*D. rotundata* (*N* = 48)				*D. alata* (*N* = 48)			
No.	Locus	*A*	*Ho*	*He*	*F*	*A*	*Ho*	*He*	*F*	*A*	*Ho*	*He*	*F*
1	YM02	3	0.000	0.653	1.000	17	0.938	0.865	−0.084	11	0.792	0.856	0.075
2	YM03	3	0.000	0.653	1.000	5	0.000	0.629	1.000	8	0.083	0.746	0.888
3	YM05	3	0.000	0.542	1.000	5	0.000	0.733	1.000	2	0.000	0.500	1.000
4	YM06	6	0.000	0.806	1.000	17	0.521	0.915	0.431	–	–	–	–
5	YM09	4	1.000	0.747	−0.340	14	0.438	0.889	0.508	8	0.000	0.764	1.000
6	YM10	4	0.000	0.736	1.000	15	0.125	0.851	0.853	21	0.229	0.928	0.753
7	YM11	3	0.000	0.625	1.000	19	0.625	0.939	0.335	–	–	–	–
8	YM12	3	0.000	0.611	1.000	3	0.000	0.559	1.000	5	0.000	0.729	1.000
9	YM13	4	0.000	0.667	1.000	8	0.000	0.807	1.000	10	0.021	0.809	0.974
10	YM21	3	0.000	0.500	1.000	18	0.542	0.927	0.416	14	0.583	0.889	0.344
11	YM23	3	0.000	0.625	1.000	19	0.426	0.922	0.539	9	0.021	0.850	0.975
12	YM24	3	0.167	0.486	0.657	9	0.229	0.816	0.719	10	0.500	0.837	0.403
13	YM32	8	0.750	0.819	0.085	15	0.479	0.902	0.469	11	0.000	0.844	1.000
14	YM33	5	0.750	0.753	0.005	15	0.438	0.911	0.520	8	0.063	0.758	0.918
15	YM36	4	0.167	0.698	0.761	10	0.271	0.762	0.645	8	0.000	0.809	1.000
16	YM37	3	0.000	0.403	1.000	12	0.174	0.790	0.780	13	0.271	0.817	0.668
17	YM44	3	0.000	0.500	1.000	12	0.542	0.900	0.398	10	0.021	0.793	0.974
18	YM45	4	0.000	0.681	1.000	20	0.271	0.914	0.704	14	0.229	0.886	0.741
19	YM53	5	0.000	0.764	1.000	17	0.104	0.929	0.888	13	0.000	0.876	1.000
20	YM55	3	0.000	0.611	1.000	15	0.229	0.910	0.748	22	0.688	0.932	0.262
21	YM65	6	1.000	0.767	−0.303	22	0.792	0.918	0.137	15	0.396	0.879	0.550
22	YM66	5	0.000	0.694	1.000	17	0.333	0.914	0.635	21	0.667	0.922	0.277
23	YM71	4	0.000	0.694	1.000	7	0.000	0.805	1.000	10	0.000	0.845	1.000
24	YM74	7	0.833	0.833	0.000	14	0.542	0.859	0.369	10	0.229	0.857	0.733
25	YM75	3	0.000	0.653	1.000	14	0.000	0.902	1.000	15	0.000	0.913	1.000
26	YM78	3	0.000	0.569	1.000	16	0.542	0.923	0.413	14	0.422	0.872	0.516
27	YM80	3	0.000	0.486	1.000	6	0.000	0.751	1.000	15	0.688	0.900	0.236
28	YM84	4	0.000	0.417	1.000	14	0.292	0.853	0.658	–	–	–	–
29	YM87	2	0.000	0.444	1.000	30	0.426	0.950	0.552	20	0.458	0.927	0.506
30	YM89	3	0.000	0.569	1.000	11	0.511	0.841	0.393	9	0.300	0.829	0.638

(mean = 0.853), respectively. In *D. alata*, 27 SSRs (90%) successfully amplified PCR products of the expected sizes that were all polymorphic. For these markers, *A* ranged from 2 to 22 (mean = 12.1), while H_o and H_e were from 0.000 to 0.792 (mean = 0.247) and 0.500 to 0.932 (mean = 0.836), respectively. The polymorphism and high level of transferability of these markers to *D. rotundata* and *D. alata* demonstrate their utility in these two economically important species globally.

To further evaluate the transferability of these markers to species outside the *D.* Sect. *Enantiophyllum* (Burkill, 1960) or the *Enantiophyllum* clade (Wilkin et al., 2005) that includes *D. cayenensis*, *D. rotundata*, and *D. alata*, we checked PCR amplification in a panel of 11 accessions of *D. dumetorum* (*D.* Sect. *Lasiophyton* or the compound-leafed clade), 2 accessions of *D. esculenta* (*D.* Sect. *Combilium* or the Birmanica clade), and 11 accessions of *D. bulbifera* (*D.* Sect. *Opsophyton* or the compound-leafed clade) (Table 4). Only nine (30%) of the selected markers detected products of the expected size in *D. dumetorum*. Of these, six (66.7%) were polymorphic, whereas all eight (26.7%) SSRs that were transferred to *D. esculenta* were monomorphic. In *D. bulbifera*, 7 (23.3%) markers could detect the correct amplicon, but only two (28.6%) were polymorphic. Overall, the transferability of the new SSRs to *Dioscorea* species beyond the *D.* Sect. *Enantiophyllum* or Enantiophyllum clade is very low.

Analysis of Genetic Relationship among *Dioscorea* Species

To test the suitability of the new SSRs for studying the genetic relationship among the major *Dioscorea* species, we selected six markers that gave amplification in at least four of the five species considered after excluding *D. esculenta* from the analysis owing to the small number of accessions (Table 4). Accordingly, a dendrogram generated on the basis of genetic distances calculated by the neighbor-joining method identified accessions at species level (Fig. 1). Consistent with the relatedness of *D. rotundata* and *D. cayenensis* and the existence of genotypes intermediate

Table 4. Transferability of the 30 selected simple sequence repeat markers to *Dioscorea* species beyond the Enantiophyllum clade. ++, polymorphic; +, monomorphic; −, no amplification; *N*, number of individuals.

No.	Locus	*D. cayenensis* (*N* = 12)[‡]	*D. rotundata* (*N* = 48)[‡]	*D. alata* (*N* = 48)[‡]	*D. dumetorum* (*N* = 12)[§]	*D. esculenta* (*N* = 2)[¶]	*D. bulbifera* (*N* = 11)[#]
1	YM002	++	++	++	−	−	+
2	YM003	++	++	++	−	−	−
3	YM005	++	++	++	−	−	−
4	YM006	++	++	−	−	−	−
5	YM009	++	++	++	−	−	−
6	YM010	++	++	++	−	−	−
7	YM011	++	++	−	−	+	−
8	YM012	++	++	++	−	−	−
9	YM013[†]	++	++	++	++	+	++
10	YM021	++	++	++	−	−	+
11	YM023	++	++	++	−	−	−
12	YM024	++	++	++	++	−	−
13	YM032	++	++	++	−	−	+
14	YM033	++	++	++	−	−	−
15	YM036	++	++	++	−	−	−
16	YM037	++	++	++	−	−	−
17	YM044	++	++	++	−	−	−
18	YM045	++	++	++	−	+	−
19	YM053[†]	++	++	++	+	+	+
20	YM055[†]	++	++	++	++	+	−
21	YM065	++	++	++	+	−	−
22	YM066[†]	++	++	++	++	−	+
23	YM071	++	++	++	++	−	−
24	YM074[†]	++	++	++	+	+	+
25	YM075	++	++	++	−	−	−
26	YM078	++	++	++	−	+	−
27	YM080[†]	++	++	++	++	+	++
28	YM084	++	++	−	−	−	−
29	YM087	++	++	++	−	−	−
30	YM089	++	++	++	−	−	−

[†] Markers used for assessing the genetic relationship among *Dioscorea* species.

[‡] *D.* Sec. *Enantiophyllum* (Enantiophyllum clade).

[§] *D.* Sec. *Lasiophyton* (the compound-leafed clade).

[¶] *D.* Sec. *Combilium* (the Brimanica clade).

[#] *D.* Sec. *Opsophyton* (the compound-leafed clade).

between the two, there was overlap in the clustering of some accessions representing these two species. Additionally, *D. alata* and *D. bulbifera* accessions both formed clusters distinct from the other species studied. However, the scattering of *D. dumetorum* accessions among Guinea yam accessions was unexpected and contradicts earlier reports that showed this species is distinct from species of the *Enantiophyllum* clade or is grouped together with *D. bulbifera* within the compound-leafed clade (Chaïr et al., 2005; Wilkin et al., 2005).

DISCUSSION

In the present study, we developed 90 *D. cayenensis* SSR markers and demonstrated their utility in multiple *Dioscorea* species. Overall, the degree of transferability of these new SSRs to other *Dioscorea* species reflected their taxonomic relationships as previously demonstrated by Tostain and colleagues (2006). The higher level of transferability to *D. rotundata* is consistent with the relatedness of these two species of African origin. Guinea yams have been described by some authors as the *D. cayenensis-rotundata* species complex (Chaïr et al., 2005; Dansi et al., 2013), while others have suggested that *D. cayenensis* is a variety of *D. rotundata* (Terauchi et al., 1992). The level of transferability of the SSRs to *D. alata*, a species of Asian origin grouped under the same *D.* Sect. *Enantiophyllum* or *Enantiophyllum* clade together with Guinea yam, is also considerable compared with the level of transferability to *D. dumetorum* (*D.* Sect. *Lasiophyton* or the compound-leafed clade), *D. esculenta* (*D.* Sect. *Combilium* or the Birmanica clade), and *D. bulbifera* (*D.* Sect. *Opsophyton* or the compound-leafed clade).

In general, the genetic relatedness of the species inferred on the basis of six selected SSRs reflected their botanical relationships (Fig. 1), indicating the potential

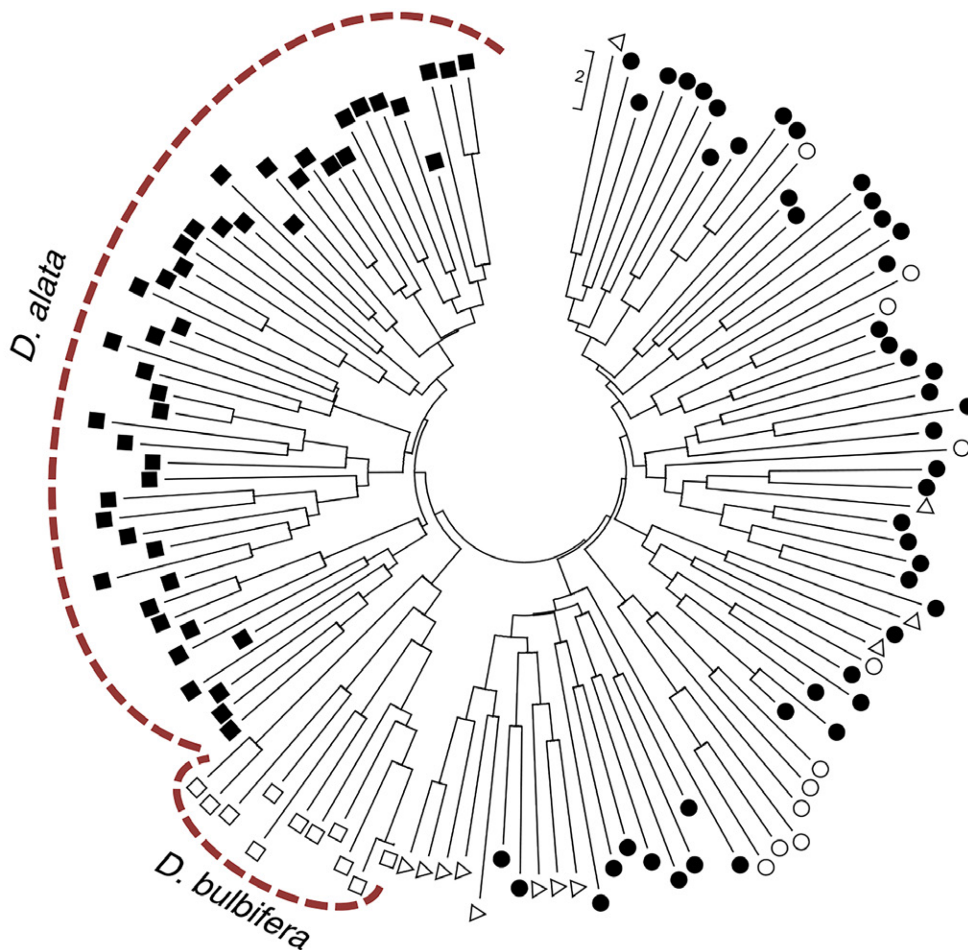

Figure 1. A dendrogram of five *Dioscorea* species based on genetic distances calculated by the neighbor-joining method. ■, *D. alata*; □, *D. bulbifera*; ●, *D. rotundata*; ○, *D. cayenensis*; △, *D. dumetorum*.

utility of these markers for the purpose of estimating genetic distance among *Dioscorea* species. However, our result that *D. dumetorum* is closely related to the Guinea yam species of *D. rotundata* and *D. cayenensis* is at odds with previous reports that grouped *D. dumetorum* separately or in the same clade with *D. bulbifera* by chloroplast DNA SSR (cpSSR) and sequence-based phylogenetic analyses (Chaïr et al., 2005; Wilkin et al., 2005). In addition to the small number of SSRs used for the analysis in this study, this finding is likely the result of the inherent nature of SSR markers. On the one hand, a major feature of SSR markers that led to their extensive application in genetic research is their extreme polymorphism resulting from high mutation rates of their sequences, which can discriminate individuals in a population from relatively few markers. Accordingly, SSRs are often isolated from a particular species of interest but used across related species or genera for applications such as genetic diversity analysis and phylogenetic construction. On the other hand, this high mutation rate can generate allelic homoplasy, which is when alleles are identical in state (or length) although not identical by descent, causing apparent similarity (Estoup et al., 2002; Barkley et al., 2009). Allele size provides an adequate measure of genetic difference in studies involving closely related individuals, but when inference of phylogenetic relationships is made among distantly related species or over longer time scale, it is important to verify the SSR allele by sequencing the SSR (Chen et al., 2002; Barkley et al., 2009). We believe that the SSR markers reported here are informative for species of the *Enantiophyllum* section or clade (*D. rotundata*, *D. cayenensis*, and *D. alata*) but will have limited practical value for species beyond this clade, particularly for making phylogenetic inferences.

Despite the importance of yam as a subsistence and cash crop for millions of people across Africa and beyond, the existing diversity both in cultivated as well as wild species remains largely uncharacterized. Their ease of use, codominance, and high levels of polymorphism make SSRs the preferred markers for characterization of genetic diversity. Additionally, as most cultivated yam species are dioecious in nature, yam breeding relies on controlled pollination to generate mapping populations, a process that is prone to contamination by illegitimate pollen, especially in an open field. Confirming the legitimacy of genetic crosses is therefore an important requirement for

genetic studies in yam. A few selected SSR markers that are polymorphic between parents used in genetic crosses can be effective for authentication of the progeny generated. We therefore believe that SSRs will continue to be the markers of choice in yam particularly for the modestly equipped national agricultural research systems that are located across the major yam-growing regions. In conclusion, the genomic SSR markers reported here provide additional public domain genomic resources for this economically important crop to serve as tools for yam genetic research, genetic diversity analysis, and selective breeding.

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