Retrotransposon molecular markers resolve cocoyam (*Xanthosoma sagittifolium*) and taro (*Colocasia esculenta*) by type and variety

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Abstract

Retrotransposon-based molecular markers were applied for the first time within the genera *Xanthosoma* and *Colocasia* to assess intraspecific variability among 27 accessions of cocoyam (*Xanthosoma sagittifolium*) and taro (*Colocasia esulenta*). Retrotransposons were isolated and sequenced; long terminal repeat (LTR) primers were designed to obtain inter-retrotransposon amplified polymorphism (IRAP) fingerprints. A set of

six chosen LTR primers yielded 433 reproducible bands across 20 *X. sagittifolium* samples. Out of the 433 bands, 400 fragments (92 %) were polymorphic. In seven *C. esculenta* accessions, the six primers amplified a total of 354 reproducible, informative data points, of which 285 (80.5 %) were polymorphic. Cluster analysis placed all the accessions in two groups according to their species. The accessions of *X. sagittifolium* were further divided into two subgroups corresponding to their ploidy level. Moreover, the genetic variability accessed by IRAP markers allowed separation of *X. sagittifolium* and *C. esculenta* accessions according to their type and botanical variety respectively. The results suggest that retrotransposon activity continued after *Xanthosoma* speciation. The data and approach provides a basis for better germplasm management, future systematic studies and genetic improvement, as well as for exploration of the role of retrotransposons in cocoyam and taro polyploid formation and genome dynamics.

AQ1

Keywords

Cocoyam
Genetic diversity
Inter-retrotransposon amplified polymorphisms (IRAP)
PBS
Taro

Abbreviations

IRAP	Inter-retrotransposon amplified polymorphism
TEs	Transposable elements
LTR	Long terminal repeat
PBS	Primer-binding site
CRRD	Cocoyam root rot disease

Data deposition The sequences reported here have been deposited in the GenBank database (accession nos.GU810535, KF813039–KF813057).

Electronic supplementary material

The online version of this article (doi:10.1007/s10681-015-1537-6) contains supplementary material, which is available to authorized users.

Introduction

The monocotyledonous *Araceae* family comprises about a hundred genera, among which five are cultivated as food crops: *Cyrtosperma*, *Amorphophallus*, *Alocasia*, *Colocasia*, and *Xanthosoma* (Purseglove 1972). The common edible aroids are found in the genera *Xanthosoma* and *Colocasia*, with *Xanthosoma sagittifolium* and *Colocasia esculenta* being the most important species. *X. sagittifolium* is an annual crop grown for its edible, corms, cormels, and leaves in the tropics and sub-tropics. It originated in the tropical Americas and spread later to Southeast Asia, the Pacific Islands, and Africa. Presently, *X. sagittifolium* is widely cultivated in subtropical and tropical regions including Central and South America, Africa, Asia, and the South Pacific islands. Consequently, *X. sagittifolium* is also referred to as tannia, new cocoyam, cocoyam, macabo, and other names depending on the place of cultivation.

X. sagittifolium is a rich source of carbohydrates, essential amino acids, vitamins, and minerals for the human diet (Sefa-Dedeh and Agyir-Sackey 2004). *C. esculenta*, or taro, is intercropped with cocoyam. The two species are phenotypically close enough to be interchangeable for preparation of many traditional West African dishes. In addition to its high nutritional value, cocoyam is progressively becoming an important cash crop, particularly for poor resource-rural populations. It is among the six most important root and tuber crops worldwide and has an annual production of about 10 million tons (FAOSTAT 2014). Despite its widespread production and importance, improved breeding and processing of cocoyam could greatly increase its utilization and value (Owusu-Darko et al. 2014).

Cocoyam root rot disease (CRRD), caused by *Pythium myriotylum*, has emerged as the main production constraint in most cocoyam growing areas worldwide (Pacumbaba et al. 1992; Perneel et al. 2006). Recommended cultural practices provide only short-term control of CRRD (Adiobo et al. 2007) and strategies to control it with biological agents are still under examination (Perneel et al. 2007). *C. esculenta* suffers great damage due to the taro leaf blight caused by *Phytophthora colocasiae* (Singh et al. 2012), but resistant varieties are available. The use of resistant varieties could be an approach to sustainable disease control, but to date no acceptable ~~disease resistant~~disease-resistant cocoyam variety has been bred. Furthermore, little is known about cocoyam plant genetics.

Generally poor knowledge of the genetic variation within the existing

cocoyam germplasm has constrained development of improved varieties. Until recently, the diversity of cocoyams, *e.g.* in Cameroon, has been assessed only with phenotypic descriptors and cytological features (Ngouo et al. 1989), resulting in the identification of three cocoyam types (White, 2n = 26; Red, 2n = 26; Yellow, 4n = 52), which were later characterized by morphology and with biochemical markers (Mbouobda et al. 2007; Onokpise et al. 1999). Efforts have been made to develop PCR-based molecular markers as well. Use of RAPD markers revealed either low (Schnell et al. 1999) or high (Offei et al. 2004) genetic variation, and no correlations were made to plant morphology; mitochondrial and chloroplast-specific restriction fragment polymorphisms revealed limited species-level variability (Brown and Asemota 2009). Microsatellite markers were recently introduced (Cathebras et al. 2014), giving very variable degrees of heterozygosity, which were observed at levels ranging from 0.00 to 0.97. Cathebras et al. (2014) were able to transfer the markers to other species of *Xanthosoma* and *Caladium* at success rates from 23.5 to 100 %. Likewise, studies using AFLP were used only to determine ~~of~~ the intergeneric relationships between *Caladium*, *Xanthosoma*, and other genera of Araceae (Loh et al. 2000). We sought a highly polymorphic marker system for cocoyam, which would be suited to low- and medium-throughput analyses, and a simple laboratory setup.

Retrotransposons have been established in many plant species as highly effective marker systems due to their ubiquity, abundance, dispersion, and genomic dynamism (Kalendar and Schulman 2006; Kalendar et al. 2011a; Schulman et al. 2012).The long terminal repeat (LTR) retrotransposons propagate within the genome through a “copy and paste” mechanism, whereby reverse-transcribed daughter copies of the retrotransposon transcripts are inserted into new locations (Schulman 2013). The LTRs, conserved within retrotransposon families, form joints with the genomic DNA at the retrotransposon insertion points and provide a means of detecting the insertions.

The retrotransposon integration joints can be detected by PCR amplification between one primer matching the LTR and another matching a nearby motif in the genome, and the polymorphic insertion sites detected by standard agarose gel electrophoresis of the PCR products (Schulman et al. 2012). For example, the inter-retrotransposon amplified polymorphism (IRAP) method amplifies segments between two nearby LTRs; ~~and~~ retrotransposon microsatellite amplified polymorphism (REMAP) amplifies segments between an LTR and a

microsatellite (Kalendar et al. 1999; Schulman et al. 2012). Retrotransposons and LTRs can be found and annotated in sequenced genomes bioinformatically. However, in uncharacterized genomes such as that of cocoyam, the LTR sequences for the development of marker methods must be isolated either by laborious walking to the LTR ends from conserved domains in retrotransposon polyproteins (Pearce et al. 1999) or by the more recently developed and rapid method known as iPBS, which is based on a highly conserved motif adjacent to LTRs (Kalendar et al. 2010).

The aim of this work was therefore to develop retrotransposon-based markers for cocoyam using the iPBS method. The LTRs isolated by iPBS were converted into IRAP primers and used as molecular markers for identifying cultivars and determining genetic relationships in *X. sagittifolium*. The transferability of the markers to the closely related araceae species, *C. esculenta* was also tested.

Materials and methods

Plant materials and DNA isolation

X. sagittifolium and taro are not native to the African continent. Much of the cocoyam cultivated in Cameroon and the rest of West Africa are landraces derived from earlier introductions especially by the Portuguese. Passport data and ultimate geographic origins of the materials are not available. The genotypes used in the experiments are listed in Table 1. Twenty different accessions of *X. sagittifolium* and seven accessions of *C. esculenta* were obtained in Cameroon from farms or from the Institute of Agricultural Research for Development (IRAD), Ekona Regional Centre, and used for the study. Accession W305 is an artificial hybrid derived from crosses between White and Red accessions of *X. sagittifolium*. Accessions RO1054 and RO2063 were selections of the ROTREP cocoyam breeding program of Cameroon.

Table 1

Analyzed accessions and their characteristics

Accession codes	Species	Ploidy	Tuber flesh color
W1	*X. sagittifolium*	2x	White
W3	*X. sagittifolium*	2x	White
W4	*X. sagittifolium*	2x	White

Accession codes	Species	Ploidy	Tuber flesh color
W7	*X. sagittifolium*	2x	White
RO1054	*X. sagittifolium*	2x	White
RO2063	*X. sagittifolium*	2x	Red
RO31	*X. sagittifolium*	2x	Red
W305	*X. sagittifolium*	2x	White
Jinika	*X. sagittifolium*	2x	White
R1	*X. sagittifolium*	2x	Red
R2	*X. sagittifolium*	2x	Red
R3	*X. sagittifolium*	2x	Red
R4	*X. sagittifolium*	2x	Red
R5	*X. sagittifolium*	2x	Red
R6	*X. sagittifolium*	2x	Red
Y1	*X. sagittifolium*	4x	Yellow
Y2	*X. sagittifolium*	4x	Yellow
Y3	*X. sagittifolium*	4x	Yellow
Y4	*X. sagittifolium*	4x	Yellow
Y5	*X. sagittifolium*	4x	Yellow
IB	*C. esculenta*	2x	
IB1	*C. esculenta*	2x	
IB2	*C. esculenta*	2x	
AS	*C. esculenta*	2x	
AS1	*C. esculenta*	2x	
AS2	*C. esculenta*	2x	
Nejet	*C. esculenta*	2x	

Following previous tests in the field for root rot susceptibility, tolerance, or resistance, these two accessions were rated as tolerant (Nyochembeng et al. 2007). Total genomic DNA was extracted from leaf samples of these genotypes using a modification of the CTAB extraction protocol (http://primerdigital.com/dna.html) with RNAse A treatment. The DNA

samples were diluted in 1 × TE buffer and the DNA quality was checked spectrophotometrically with a Nanodrop apparatus (Thermo Fisher Scientific Inc.) as well as electrophoretically.

Isolation of retrotransposon fragments

The iPBS method (Kalendar et al. 2008, 2010) was used to obtain retrotransposon fragments containing LTR regions from *X. sagittifolium*. The method is based on PCR amplification between the primer binding sites (PBS) that serve in minus-strand cDNA synthesis, which are highly conserved in virtually all retrotransposons and located adjacent to the 5′ LTR (Kalendar et al. 2010). The PCR amplification was performed with one or two primers complementary to particular PBS types. A total of 96 PCR fragments were isolated and cloned into the vector pGEM-T (Promega, USA), then sequenced using an ABI3700 (Applied Biosystems, USA) capillary sequencer.

Retrotransposon segments were recognized by a combination of approaches: alignment of the sequenced PCR fragments to identify conserved repetitive segments; comparison of these segments to LTRs and internal regions of annotated retrotransposons; identification of the diagnostic structural features of retrotransposons, including the inverted terminal repeats of LTRs containing the terminal nucleotides 5′ TG…CA 3′ and the 3′ terminus of the 5′ LTR being positioned one to five nucleotides away from the PBS. Primers were designed with the FastPCR software (Kalendar et al. 2011b), to match the conserved segments of retrotransposon LTRs in various orientations.

IRAP reactions

IRAP analysis was carried out according to Kalendar and Schulman (2006). PCR reactions for IRAP were performed in 25 µl containing 25 ng genomic DNA, 1 × DreamTaq buffer, 0.3 µM each primer, 0.2 mM each dNTP, and 1 U DreamTaq DNA polymerase (Thermo Scientific). Amplifications were performed in a Mastercycler Gradient (Eppendorf AG, Germany) in 0.2 ml tubes or in 96-well plates. After an initial denaturation step at 95 °C for 3 min, thermocycling was performed for 32 cycles at 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 90 s, with a final extension at 72 °C for 5 min.

Each primer was tested individually in PCR reactions using a genomic DNA mixture composed of equal amounts from each of the accessions. The PCR products were separated by electrophoresis at 60 V for 8 h in a 1.4 % agarose gel (RESolute Wide Range, BIOzym) with 0.5 × TBE electrophoresis buffer.

The Thermo Scientific GeneRuler DNA Ladder Mix, 100-10,000 bp, #SM0332, was used as a standard. Gels were stained with EtBr and scanned using a FLA-5100 imaging system (Fuji Photo Film GmbH) at a resolution of 50 μm.

IRAP fingerprint analyses

From the IRAP profiles, all distinct bands were scored as present (1) or absent (0) at each band position for each primer in the 27 samples. Each IRAP band was treated as a single locus. The presence or absence of a fragment of a given length was recorded in binary code. The sets that contained missing values were removed from the raw scored data sets. Monomorphic bands were scored and removed from the data set before analysis.

The 50 IRAP primers were tested for the number, distinctness, robustness, and ease of scoring of the fingerprint bands (Table 2). Six primers generating reproducible and robust patterns were retained for subsequent analysis. The discriminatory power of these six primers was evaluated by means of the number of bands generated and by the following two parameters. The polymorphic information content (PIC) for each primer was calculated using the online program PICcalc (Nagy et al. 2012). The effective multiplex ratio (the number of polymorphic products from a single amplification reaction) was calculated according to Powell et al. (1996).

Table 2

IRAP primers: their retrotransposon source, sequence and efficiency in single-prim amplification as tested on *X. sagittifolium* and *C. esculenta* accessions

Primer[a]	Retrotransposon GenBank accession, primer location	Primer sequence (5′–3′)	IRAP efficacy
2950	GU810535, 53←74	AGGATGGGTGACCTTCCTGGGA	5
2951	GU810535, 7←28	GGGTCAGTTAGCTGATTGGGTC	5
2952	GU810535, 52→73	TTCCCAGGAAGGTCACCCATCC	5

[a]Primers selected for further analyses are underlined

[b]PCR efficacy rating scale: *0* no bands, *1* few and weak bands, *2* a few strong bands, *3* ≥10 strong bands, *4* many bands (good primer), *5* many strong and equally amplified bands (excellent primer)

Primer[a]	Retrotransposon GenBank accession, primer location	Primer sequence (5′–3′)	IRAP efficacy
2953	GU810535, 139→159	ACTAGTCCGGGTTGTTGGTAG	5
2954	KF813039, 74←97	GCTTCGTGCTTGCTGTTTGGAGTG	4
2955	KF813039, 253←276	TGTGGTGCCTTTGCACGGTGTTCC	3
2956	KF813039, 264→284	CAAAGGCACCACACGCTCCAC	4
2957	KF813039, 847→867	TCCTCGAAGAGCCTCCACTCC	4
2958	KF813040, 1←22	TAGTCGGAGCCGGGGCGTGACA	4
2959	KF813040, 7→27	GCCCCGGCTCCGACTACGGCA	5
2960	KF813040, 56→76	GTCAGAACCGGAACGCGACAG	5
2961	KF813040, 194→220	CCCAAAGACCGCACATAAATATTTACA	2
2962	KF813041, 13←33	GCTCGGACATCTTGTTCACGA	2
2963	KF813041, 383←406	GTCCACTCGAGGCGCTTGGGTGAC	2
2964	KF813041, 46→66	AGAACATGGAGGTGTACGTGG	5
2965	KF813041, 396→418	CTCGAGTGGACGCCGAGCTGCCA	4
2966	KF813056, 298→322	AACACTCTACAACCGGCTCTACTCA	4
2967	KF813056, 229←251	CTTGCGAGGAGCAAGGCCGAACC	4
2968	KF813054, 264←284	TATACGGGTCATTACGATCCG	2
2970	KF813054, 29→49	GATGATGCAGGGACTGCTGAC	4

[a]Primers selected for further analyses are underlined

[b]PCR efficacy rating scale: *0* no bands, *1* few and weak bands, *2* a few strong bands, *3* ≥10 strong bands, *4* many bands (good primer), *5* many strong and equally amplified bands (excellent primer)

Primer[a]	Retrotransposon GenBank accession, primer location	Primer sequence (5′–3′)	IRAP efficacy
2971	KF813054, 34←54	TCCTAGTCAGCAGTCCCTGCA	5
2972	KF813056, 71→94	GGGGTTGAATAAATCAGCCCCAAA	4
2973	KF813056, 29←52	CCTTAGATCCGATCCCCTCTTTTA	0
2974	KF813048, 753→773	CCCTAGTCCAGATCCCCTACC	4
2975	KF813048, 707←729	ATCCACCCCCAAGGAAATGACCA	4
2976	KF813049, 330→350	CTGAGTCACCCCGTGGCTTTT	1
2977	KF813049, 331←351	CAAAAGCCACGGGGTGACTCA	5
2978	KF813049, 108←128	TCGGTTTCCAGCCACCACCAA	5
2979	KF813053, 28←48	CTCTGTGACGCAACCTGGGAC	5
2980	KF813053, 37→58	TGCGTCACAGAGGGACAACCCT	5
2981	KF813050, 407→427	CGAGTTTCTTCTTGAGTCCCA	4
2982	KF813050, 394←414	GAAACTCGAGGGAGGGCATCT	5
2983	KF813051, 26←48	ACCTGTGCTTCCGGGGCGTTACA	3
2984	KF813051, 43→63	ACAGGTACAGCACCAATGGCT	5
2985	KF813051, 448→469	TGACTCAGTAGGAACCACATCA	4
2986	KF813043, 113→134	CGGTAGCGTTCTTGGCCATCGA	5

[a]Primers selected for further analyses are underlined

[b]PCR efficacy rating scale: *0* no bands, *1* few and weak bands, *2* a few strong bands, *3* ≥10 strong bands, *4* many bands (good primer), *5* many strong and equally amplified bands (excellent primer)

Primer[a]	Retrotransposon GenBank accession, primer location	Primer sequence (5′–3′)	IRAP efficacy
2987	KF813043, 66←86	TCTTGGTAGCGTCCATCGGAC	0
2988	KF813055, 23←43	CTTCTCCCTGCTGCAATTTCC	3
2989	KF813055, 38→58	GAGAAGACGCGGTTGCACAGA	4
2990	KF813047, 275→295	CTCCTCTGAGGCCTTGAAAGC	3
2991	KF813047, 210←231	CCGGCTATACCTTGGGGACCCA	5
2992	KF813046, 412→433	GGCCGTACTCATCTGGGCAACA	5
2993	KF813046, 140←161	CAAACCCTCAGCCGGTTTACAC	4
2994	KF813052, 481→504	TGGCTTCGTATAATGCTGTGCCGT	4
2995	KF813052, 481←502	GGCACAGCATTATACGAAGCCA	0
2996	KF813057, 21←44	GATTATACATCTCTTGACCTAACA	4
2997	KF813045, 459→479	GGTCTGACGGAAGTTCACTGC	4
2998	KF813042, 883→904	TGAGTTCCCTCCTCCGGCAAGA	4
2999	KF813042, 715←737	CACACCCCAGAACAACGAGCAGC	5
3000	KF813044, 515→535	CGAGGTGCTGAGGCACTTCAG	3

[a]Primers selected for further analyses are underlined

[b]PCR efficacy rating scale: *0* no bands, *1* few and weak bands, *2* a few strong bands, *3* ≥10 strong bands, *4* many bands (good primer), *5* many strong and equally amplified bands (excellent primer)

Several software programs were then used to analyze the data. Genetic similarity was evaluated using PopGene software version 1.32 (Yeh and Boyle 1997) for the Shannon index (H'j; Shannon and Weaver 1949), which is

defined for multilocus markers as: $H'_j = -\sum p_i \log p_i$, where pi is the frequency of the ith fragment in the sample. In addition, the genetic similarity index of Jaccard (1908) was used to calculate genetic similarity, employing the software GenStat Discovery 4 (2012) and FAMD (Schlüter and Harris 2006). Given two genotypes, A and B, the Jaccard similarity index J is defined as: $J = M_{11}/M_{01} + M_{10} + M_{11}$, where M_{11} is the total number of bands both having a value of 1, M_{01} is the total number of bands whose values are 0 in A and 1 in B, and M_{10} is the total number of bands whose values are 1 in A and 0 in B. The average Jaccard indices were calculated separately for data obtained on *X. sagittifolium* and *C. esculenta* accessions.

In order to compare levels of diversity detected by different primers, the various parameters were calculated for each primer separately. One-way ANOVA and Tukey's test were performed on genetic similarity indices. Principal component analysis (PCA) using the GenAlex 6.5 program (Peakall and Smouse 2006, 2012) was carried out to define the relationships among *X. sagittifolium* types (White, Red and Yellow) and among *C. esculenta* varieties (*C. esculenta* var. *esculenta* and *C. esculenta* var. *antiquorum*), based on the data from all six chosen primers. A pairwise genetic distance matrix was calculated, then converted to a covariance matrix and used to perform the PCA.

The ability of IRAP markers to reveal genetic relationships among all *X. sagittifolium* and *C. esculenta* accessions and between *X. sagittifolium* and *C. esculenta* accessions was evaluated phylogenetically by Neighbor-Joining (NJ). An algorithm was constructed using PAUP software (Swofford 1998). Support for the tree was determined by performing 1000 bootstrap operations on the data set generated by distance analysis. To study the partition of IRAP genetic variation into among- and within-population variance components, an analysis of molecular variance (AMOVA) was conducted with the program GenAlex 6.5 (Peakall and Smouse 2006, 2012). The genetic distances among groups were analyzed with the Phi statistic (Φst). To test the significance of the genetic distance among groups, AMOVA was used with 999 permutations. The accessions of *X. sagittifolium* were defined as diploid (White and Red types) or tetraploid (Yellow type).

Results

LTR isolation

Retroviruses and retrotransposons prime minus-strand reverse transcription,

an essential step of replication, with specific tRNAs of the host cell (Schulman 2013). The PBS site is complementary to the tRNA primer and adjacent to the 5′ LTR. The iPBS method exploits primers matching common PBS motifs as a means of amplifying segments of two LTR retrotransposons in close proximity to one another. To screen the cocoyam genome for retrotransposons, a set of 83 PBS primers were used in iPBS. Most produced multiple bands, expected for abundant and clustered retrotransposons. A total of 96 iPBS PCR reaction products, ranging from 400 to 2000 bp long, were cloned, sequenced, and aligned, identifying clusters of highly similar fragments. A total of 93 clusters were identified, which have features typical for LTRs (Benachenhou et al. 2013; Schulman 2013); 22 LTRs were deposited in Genbank (accessions GU810535, KF813039-KF813057). Primers were designed to match these (Table 2), positioned close to the predicted ends of the LTRs and oriented outwards from the LTRs.

Initial screening of primers

The IRAP protocol was used to screen the 50 primers for their ability to produce polymorphic patterns across 27 accessions of *X. sagittifolium* and *C. esculenta*. The majority of primers amplified bands which were easy to score on standard agarose gels stained with ethidium bromide. The PCR reactions produced variable numbers of amplified fragments depending on the primer tested (Table 2). Only four primers (2969, 2973, 2987 and 2995) produced no bands. Altogether 18 primers gave superior results, with many strong and equally amplified bands. Six of these (2952, 2964, 2971, 2977, 2978, 2979) were selected for evaluation of genetic diversity in the accessions and for further analysis.

Genetic diversity in *X. sagittifolium* accessions

The IRAP protocol was used to study genetic variability among 20 accessions of White, Red, and Yellow cocoyam using the six chosen primers singly in PCR reactions (Table 3; Fig. 1). A total of 433 reproducible bands, ranging from 400 bp (primer 2950) to 3000 bp (primer 2950) were detected using the six selected primers. The number of bands per primer varied from 60 (primer 2952) to 86 (primer 2977). Of the 433 bands, 400 fragments (92 %) were polymorphic across the whole set of samples (Table 3). The primers displayed between 82 % (primer 2952) and 97 % (primer 2977) polymorphism across the samples analyzed. Based on the Jaccard index, a genetic similarity matrix was constructed using the IRAP data to assess the genetic relatedness among the

20 *X. sagittifolium* accessions. The minimum genetic similarity (0.23) was between R5 and Y4 and the greatest (0.85) between R1 and R3. Genetic similarity coefficients indicating the extent of relatedness between each pair of accessions are presented in Supplementary Table S1 and ANOVA for the data of all six primers taken together are found in Table S2.

Table 3

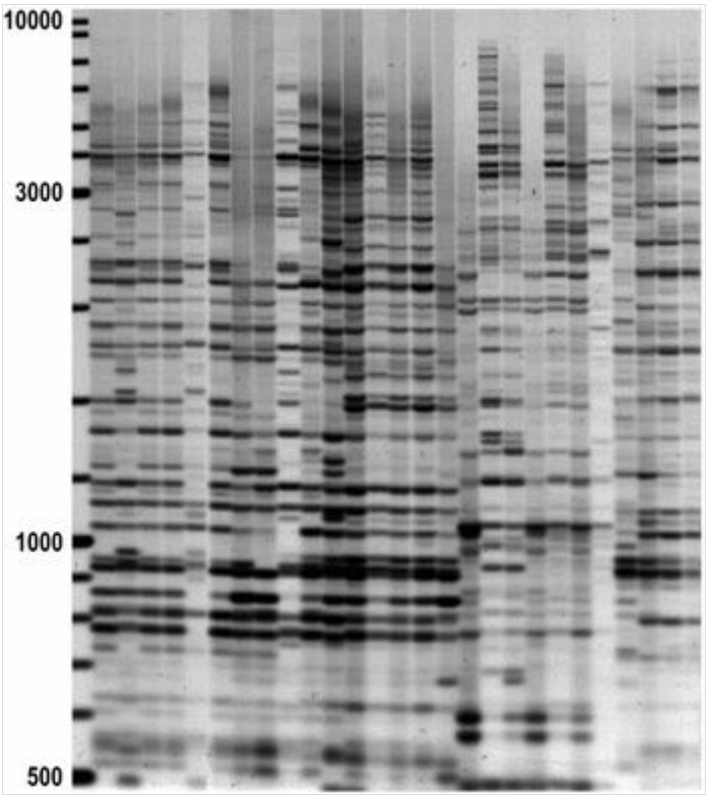
Band numbers and polymorphism measures in 20 *X. sagittifolium* and seven *C. esculenta* accessions, for selected primers

Accessions	Primers						Mean (±SE)
	2952	2964	2971	2977	2978	2979	
X. sagittifolium							
Number of bands	60	78	82	86	70	57	
% Polymorphic loci	88.33	91.02	93.9	96.51	88.57	94.73	
Shannon index	0.499	0.518	0.458	0.469	0.519	0.501	0.494 ± 0.010[a]
Average Jaccard index	0.475	0.475	0.424	0.436	0.55	0.459	0.469 ± 0.018[a]
C. esculenta							
Number of bands	50	60	70	70	57	47	
% Polymorphic loci	82	80	90	82.85	57.89	89.36	
Shannon index	0.498	0.493	0.53	0.536	0.469	0.526	0.508 ± 0.010[a]
Average Jaccard index	0.499	0.519	0.521	0.544	0.482	0.513	0.447 ± 0.016[a]

[a]Listed in Table 2

Fig. 1

Agarose gel electrophoresis of IRAP reactions using the primer 2971for *X. sagittifolium* accessions. *Lanes*, *left* to *right*: 1, size ladder (bp); 2, W1; 3, R6; 4, W3; 5, W4; 6, Y5; 7, not analyzed; 8, W7; 9, RO1054; 10, RO2063; 11, RO31; 12, W305; 13, Jinika; 14, R1; 15, R2; 16, R3; 17, R4; 18, R5; 19 IB; 20, IB1; 21, IB2; 22, AS; 23, AS1; 24, AS2; 25, Nejet; 26, Y1; 27, Y2; 28, Y3; 29, Y4



Genetic diversity in diploid and tetraploid *X. sagittifolium* accessions

In a different analysis, IRAP was used to study the relationship between ploidy level and cocoyam type using the six primers. Four White diploids (W1, W3, W4 and W7), five Red diploids (R1, R2, R3, R4 and R6), and five yellow tetraploids (Y1, Y2, Y3, Y4 and Y5) were examined. The selection of these accessions was based on their having a known ploidy level and on their grouping in the dendrogram. Within the *X. sagittifolium* accessions analyzed, the number of bands scored was higher for tetraploids than for diploids: 278 for the White diploids, 301 in Red diploids, and 367 for Tetraploids. In parallel, the number of bands for individual primers varied from 35 (primer 2979, Red diploids) to 74 (primer 2977, tetraploids). Primer 2977 used with tetraploid accessions showed the highest number of polymorphic (56) and private (23) bands (Table 4). The total number of polymorphic loci showed a pattern similar to the total scored, from 101 (White diploids) to 147 (Red diploids) and 259 (Tetraploids), as did the number of private bands exclusively present in accessions of only one of the genome groups, respectively 9, 14, and 72 for White, Red, and Tetraploids, with respectively three, four, and twelve being monomorphic.

Table 4

IRAP analysis of four White diploids, five Red diploids, and five tetraploid accessions of *X. sagittifolium* using the six selected primers

Accession types	Primers						Total
	2952	2964	2971	2977	2978	2979	
White type (diploid)							
Number of bands	43	50	46	49	51	39	278
Number of polymorphic bands	13	25	13	23	12	15	101
Number of private bands	1	0	2	2	1	3	9
Number of fixed private bands	1	0	0	0	0	2	3
Red type (diploid)							
Number of bands	40	63	57	51	55	35	301
Number of polymorphic bands	17	38	31	22	17	22	147
Number of private bands	1	3	3	3	1	3	14
Number of fixed private bands	1	0	1	1	1	0	4
Yellow type (tetraploid)							
Number of bands	54	70	63	74	61	45	367
Number of polymorphic bands	36	54	45	56	39	29	259
Number of private bands	10	9	12	23	9	9	72
Number of fixed private bands	2	1	1	3	1	4	12

Genetic variability based on IRAP analysis in *C. esculenta*

The IRAP method was used to study genetic variability among different accessions of *C. esculenta* using the same primers developed for *X. sagittifolium*. The PCR reactions produced a high number of bands for all IRAP primers. As shown in Table 3, the six primers amplified a total of 354 reproducible and informative data points, of which 285 (81 %) were polymorphic, in the set of seven *C. esculenta* accessions. Between 47 (primer

2979) and 70 (primers 2971 and 2977) bands, varying from 300 to 3000 bp, were produced per primer. These products were from 58 % (primer 2964) to 90 % (primer 2971) polymorphic. The IB and Nejet accessions showed the lowest genetic Jaccard index (0.10), whereas the highest (0.63) was observed between IB and AS. Genetic similarity coefficients indicating the extent of relatedness between each pair of *C. esculenta* accessions are presented in Supplementary Table S3.

X. sagittifolium versus *C. esculenta* variability

Overall, also *C. esculenta* genotypes exhibited a high level of genetic diversity, even though their fingerprints contained generally fewer bands than those of *X. sagittifolium* accessions for all six primers. The Shannon index was on average 0.494 (range 0.458–0.519) for *X. sagittifolium* and 0.508 (range 0.469–0.536) for *C. esculenta* genotypes (Table 3). The Jaccard index shows a similar trend (Table 3), but there is no significant difference for these indices between the two groups. The lowest PIC scores (respectively 0.364 and 0.387) were obtained respectively with primer 2978 for *X. sagittifolium* accessions and primer 2971 for *C. esculenta* accessions. The remaining PIC values for the six primers were almost similar and ranged between 0.372 and 0.374 for the two groups of accessions (Table 5). The effective multiplex ratio (EMR) values (Table 5) for *X. sagittifolium* accessions ranged from 46.81 to 80.10 (respectively primers 2952 and 2977), whereas those for *C. esculenta* ranged from 37.53 to 56.7 (primers 2979 and 2971).

Table 5

Primers and their polymorphism information content (PIC) and effective multiplex ratio (EMR) values generated from *X. sagittifolium* and *C. esculenta* accessions

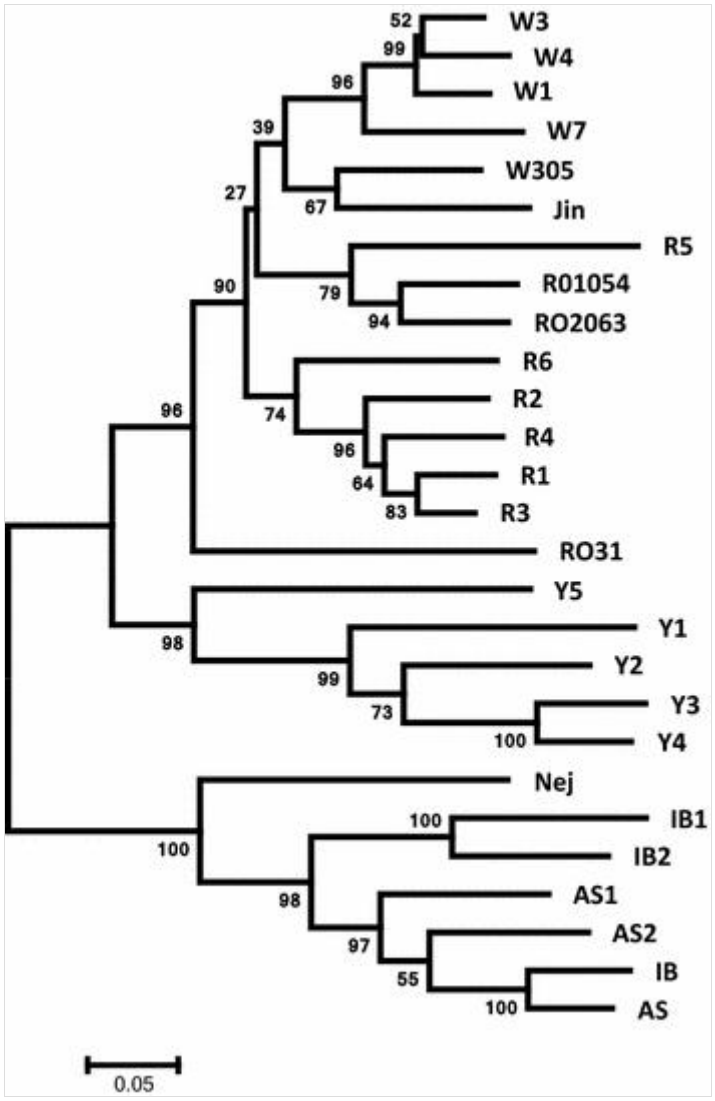
	Primers					
	2952	**2964**	**2971**	**2977**	**2978**	**2979**
X. sagittifolium						
PIC	0.373	0.374	0.373	0.373	0.364	0.374
EMR	46.81	64.62	72.30	80.10	54.91	51.15
C. esculenta						
PIC	0.374	0.374	0.367	0.372	0.372	0.374
EMR	33.62	38.4	56.7	48.05	19.10	37.53

Multivariate analyses

Phylogenetic relationships among the 27 accessions of cocoyam and taro were estimated by NJ according to Nei's genetic distance matrices. In the resulting dendogram (Fig. 2), the two species each produce a major cluster. The accessions of *X. sagittifolium* were further divided into two well-supported clusters that are consistent with the cytogenetic genome groups, the tetraploids (4n = 52) and the diploids (2n = 26). The diploid cocoyam accessions were further clustered into almost type-specific subclusters resolving the White and Red cocoyams. However, three accessions, two Red (RO2063 and R5) and one White (RO1054), did not cluster with their types. Interestingly, accessions W305, an artificial hybrid, and Jinika, apparently a natural hybrid, clustered with the white types. The second major cluster, comprising *C. esculenta* accessions, resolved two subgroups representing two subspecies, Nejet for *C. esculenta* var. *esculenta* and. the six remaining accessions for *C. esculenta* var. *antiquorum*.

Fig. 2

Neighbor-joining dendrogram ~~of~~ showing the genetic relationships among 20 accessions of *X. sagittifolium* and 7 accessions of *C. esculenta* based on IRAP analysis. Percent support from 1000 bootstrap operations is shown



When the combined IRAP data of the six primers was subjected to PCA analysis (Supplementary Fig. S1), the tetraploid Yellow *X. sagittifolium* type was separated from the White and Red accessions by the first three principal components (47.85 % of the diversity). Most of the Yellow accessions were well resolved by Coordinate 3 alone. The hybrids W305 and Jinika, respectively White and Red, were closer to the Red accessions by Coordinate 1 and intermediate between White and the Red accessions by Coordinate 2. For the taro accessions (Supplementary Fig. S2), the first two components accounted for 58.41 % of the variation and PCA separated accession Nejet (*C. esculenta* var. *esculenta*) from the accessions belonging to the *antiquorum* subspecies. The accessions are widely scattered, confirming the large variability among them.

Analyses of molecular variance (AMOVA) revealed that a highly significant genetic differentiation occurred in the *X. sagittifolium* types (Supplementary Table S4). At two hierarchical levels, most of the genetic variation (67 %) could be attributed to differences within the populations (i.e., within White,

Red, and Yellow types) and only 33 % to differences among the populations. Pairwise Φst values (Supplementary Table S5) were obtained based on the AMOVA analysis and showed that the greatest genetic distances are between White and Yellow types (0.439) and the smallest between White and Red types (0.189), with the distance between Red and Yellow types being intermediate (0.368).

Discussion

Most recent advances in the fields of genomics and molecular genetics have been aimed at those crops, particularly the major cereals, that sustain the economy and ensure food security primarily in the developed world. However, crops of similar potential importance to the developing world are still understudied. *X. sagittifolium* (cocoyam) and *C. esculenta* (taro) are important West African food crops. Currently, molecular markers have been only limitedly applied to cocoyam, the genetic variation of which is poorly characterized; both cocoyam and taro are classified as orphan crops by the African Orphan Crops Consortium (http://www.mars.com/global/african-orphan-crops.aspx). No acceptable disease-resistant cocoyam variety has been bred. In this work, we have taken advantage of the ubiquity and abundance of LTR retrotransposons in plant genomes and their role in genomic diversification to develop and apply retrotransposon marker systems for cocoyam and taro, to our knowledge for the first time.

Cocoyam genotyping by retrotransposon markers

Using the iPBS method (Kalendar et al. 2010), we cloned and sequenced 92 PCR fragments, from 400 to 2000 bp, representing the first identified retrotransposon segments from *Xanthosoma*. When converted into IRAP primers, the majority displayed sufficiently high levels of polymorphism to be generally applicable as molecular markers. The 6 most informative primers were applied to 20 *X. sagittifolium* accessions and yielded a high number of bands. By comparison, RAPD markers used for *Xanthosoma* spp. genotypes (Schnell et al. 1999; Offei et al. 2004) showed low numbers of amplified bands. The abundant IRAP and iPBS bands may indicate that retrotransposons are abundant in the *Xanthosoma* genome or they are predominantly clustered, or both. The *X. sagittifolium* genome has been reported at 8592 Mbp (Ghosh et al. 2001). Even if, for the sake of argument, we assume a relatively high gene number of 40,000 of average length 5000 bp, the total gene space would comprise only 200 Mbp of the genome. The remaining 98 % would therefore

comprise repetitious sequences, the great majority of which ~~composed of~~being retrotransposons. The abundance of iPBS and IRAP bands amplified from the *X. sagittifolium* is consistent with this perspective.

Impact on *X. sagittifolium* characterization and taxonomy

We investigated whether the three cocoyam groups defined by morphological and cytological features (Ngouo et al. 1989; Onokpise et al. 1999) can also be differentiated by the retrotransposon genomic insertion patterns revealed by IRAP markers. Indeed, a set of six IRAP markers separated the ~~yellow~~ Yellow tetraploid accessions from the diploid White and Red ones. The hybrid accessions displayed an intermediate position in PCA based on their IRAP banding patterns. Moreover, IRAP markers distinguished individual accessions, which could not be achieved by RAPD markers (Offei et al. 2004).

A broader problem related to germplasm and biodiversity conservation of cocoyam is the unclear taxonomic position of cultivated *Xanthosoma*. The tendency has been to classify most edible, tuber bearing members of the genus as *X. sagittifolium* (Giacometti and Leon 1994). However, using leaf shape, pigmentation, and other vegetative characteristics, Wilson (1984) identified several species, including *X. violaceum*, *X. caracu*, and *X. atrovirens*. Bown (2000) divides the genus into two main species based on the color of the corm, cormels, and leaves and on the shape of the cormels. The results from IRAP analyses reported here support the splitting of cultivated *Xanthosoma* into several species. Further insight into the genetic diversity of cocoyams therefore would be gained from analysis of all species belonging to *Xanthosoma* by retrotransposon-based markers. A well-defined and established systematics of *Xanthosoma* would enable better management of germplasm collections and facilitate clonal selection.

Tetraploidy and IRAP polymorphism

The tetraploid accessions on average showed more polymorphic IRAP bands than did the diploids. The greater number of bands in the tetraploids may be due to their having more retrotransposon insertion loci. The increased polymorphism may be explained in several ways. The “genome shock” (McClintock 1984) of polyploidization has been reported to activate the transcription (Kashkush et al. 2003) and propagation (Kraitshtein et al. 2010; Parisod et al. 2010) of retrotransposons. Alternatively, the redundancy of the

gene complement in polyploids, especially autopolyploids, could reduce the selective pressure against retrotransposon insertions into genes and thereby lead both to loss of fewer retrotransposon insertions by purifying selection and to fixation of more (Hazzouri et al. 2008). A third explanation is that the increased IRAP polymorphism of tetraploids reflects an increase in overall genetic diversity in polyploids (Wendel 2000) in general and cocoyam in particular (Ngouo et al. 1989). Last, shrinkage of genomes subsequent to polyploidization (Grover et al. 2008) may lead to differential loss of retrotransposon insertions in various populations and increased polymorphism as detected by marker methods. All four scenarios are consistent with the results obtained.

Implications for cocoyam breeding

The IRAP method resolved cocoyam accessions well. The two hybrids could also be distinguished from the parental sets and grouped together. Furthermore, IRAP markers enabled detection of accessions with similar physiological traits. For example, RO1054 and RO2063, which are tolerant to disease (Nyochembeng et al. 2007), clustered together in the dendogram even though they are morphologically different. This result also indicated that the morphological system may be useful for morphotype management but not appropriate for defining genetic diversity in cocoyam.

One of the most important problems in *X. sagittifolium* production is the susceptibility to CRRD caused by the fungus *P. myriotylum*. In response, efforts are being made to broaden the genetic base of breeding populations to allow for selection of resistant lines. However, acceptable cocoyam varieties resistant to CRRD are not yet available, despite considerable advances including the creation of hybrids and mutants, the introduction of polyploids, and study of interactions between cocoyam and *P. myriotylum* (Boudjeko et al. 2005; Nyochembeng et al. 2007). Because intervarietal hybridization is a potential means to genetically improve *X. sagittifolium* for major agronomic traits and for resistance to CRRD, IRAP markers are an important molecular tool that may assist cocoyam breeders when applied to map QTLs or major genes in mapping crosses or for marker-assisted selection. The resistance of Yellow cocoyam to disease has been attributed partly to its tetraploid nature; therefore, it has been proposed that inducing polyploidy in White and Red diploid types will improve their resistance (Tambong et al. 1998; Doungous et al. 2011). IRAP markers developed in this work can assist cocoyam breeders selecting desired genotypes following polyploidy induction treatments.

Suitability of *X. sagittifolium* IRAP markers in *C. esculenta*

Here, we assessed the usefulness of *X. sagittifolium*-derived retrotransposon markers to investigate genetic variability and relationships among seven *C. esculenta* accessions, and observed a high level of polymorphism (80.5 %). This is the first report to our knowledge of genetic relationships in taro using retrotransposon markers. The *X. sagittifolium* and *C. esculenta* accessions that were examined were well separated into two distinct groups by IRAP markers, as others had been earlier by RAPD (Brown and Asemota 2009). However, both RAPD (Irwin et al. 2008) and AFLP methods (Quero-Garcia et al. 2004) failed in some cases to resolve or reflect geographical origin or morphological characters in taro. Moreover, the IRAP markers proved to be powerful enough to differentiate even the two botanical varieties (*esculenta* and *antiquorum*), although RAPD markers could not (Irwin et al. 1998). Confirmation of the utility of IRAP on taro will require, however, a wider set of material to be examined.

AQ2

The genetic separation of *X. sagittifolium* from *C. esculenta* accessions supports the suitability of IRAP for differentiating between closely related taxa of Araceae. In contrast, the AFLP fingerprint patterns based on primers effective for *C. esculenta* were too dissimilar to those of *X. sagittifolium* and other closely related species to include them in parallel diversity analyses (Kreike et al. 2004). In previous studies, IRAP has been used successfully for assessing genetic identities and relationships of closely related species (Antonius-Kemola et al. 2006) as well as in the studies of genetic diversity in *Helianthus* (Vukich et al. 2009) and *Linum* (Smykal et al. 2011). Despite the use of IRAP primers derived from *X. sagittifolium* retrotransposons, the number and polymorphism of IRAP products in *C. esculenta* was high for all six primers. This suggests that both species may share active and conserved families of retrotransposons as ~~reported~~ described previously for the grasses (Vicient et al. 2001) and confirms the inter-generic transferability of retrotransposon markers previously reported in *Spartina* (Baumel et al. 2002) and *Crocus* (Alavi-Kia et al. 2008).

Conclusion

We demonstrate that the IRAP marker system provides a simple and useful approach for studying genetic diversity of cocoyam and taro. The high percentage of polymorphism and the large number of bands obtained per assay

using a single primer shows that IRAP is the most informative marker system so far developed for *X. sagittifolium*. It was possible to identify cocoyam accessions including hybrids, diploid, and tetraploid plants and accessions that are susceptible or tolerant to the CRRD based on the marker pattern generated by six IRAP primer sets. Furthermore, the primers could also be used for *C. esculenta* and were successful in identifying different *C. esculenta* subspecies. The results obtained in this study could be very useful in the future for defining germplasm conservation and designing cocoyam and taro improvement programs for the development of disease resistant varieties.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest. The experiments comply with the current laws of the countries in which they were performed.

Electronic supplementary material

Below is the link to the electronic supplementary material.

Supplementary material 1 (PPTX 253 kb)

Supplementary material 2 (DOCX 26 kb)

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