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Research Communication

Genetic Analysis of Sweetpotato and Wild Relatives using Inter-simple Sequence Repeats (ISSRs)

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Inter-simple sequence repeat (ISSR) amplification was evaluated for its usefulness in generating DNA markers for sweetpotato and related wild species. ISSR markers were obtained through PCR amplification using simple sequence repeat (SSR) primers. Optimization of the reaction conditions was successfully achieved for 24% out of 100 SSR primers screened. The functional primers included anchored and unanchored primers. All of the anchored primers used were dinucleotide repeat motifs. The unanchored primers consisted of tri-, tetra- and penta-nucleotide repeat motifs. Eight primers were selected and employed to assess the genetic diversity and relationship between 34 accessions of sweetpotato and its related wild species. The ISSR markers are highly polymorphic among the taxa studied. Several sweetpotato accessions were clustered together based on their geographic origin. The mode of inheritance of the ISSR markers was analyzed in a pseudo-test cross mapping population. Twenty-two SSR primers amplified 70 reproducible polymorphic ISSR markers and Mendelian segregation of polymorphic bands was demonstrated. Among the 70 ISSR markers, simplex and duplex markers accounted for 70% and 15.7%, respectively. One linkage between two simplex ISSR markers was found. Thus, the ISSR markers could be suitable for fingerprinting of cultivated sweeptotato and its related wild species and for constructing a linkage map of sweetpotato.

Key Words: ***Ipomoea*, ISSR, genetic relationship, inheritance, linkage.**

Introduction

Sweetpotato (*Ipomoea batatas* (L.) Lam) is the world's seventh most important food crop after wheat, rice, maize, potato, barley and cassava (Huamán 1999). It produced a stable yield under a wide range of environmental conditions and is cultivated in more than 100 countries. Sweetpotato is widely used as food and animal feed, and is processed into snacks, starch, liquor, flour and a variety of other industrial products. In addition, sweetpotato is considered to be a food security-crop due to its long history of saving lives during famines (Diaz *et al.* 1996). Despite its importance, there have been few genetic studies on sweetpotato, probably because of its self-incompatibility and a high level of cross-incompatibility, ploidy level (hexaploid) and large chromosome number (2n = 6X = 90).

Nearly 26,000 accessions of *Ipomoea* species are maintained at various genebanks in the world and 8,000 accessions are sweetpotato cultivars or breeding lines (Kuo 1991, Rao *et al.* 1994). Wild species of *Ipomoea* are an important reservoir of useful genes and may provide a new approach for genetic improvement (Komaki 2001). However, the taxonomic relationships of sweetpotato and its related wild species have not been fully elucidated. Therefore, it is important that we understand the extent of genetic diversity and the nature of genetic relationships among sweetpotato and its related species (He *et al.* 1995).

ISSRs (inter-simple sequence repeats) are the regions between two microsatellites (also called simple sequence repeats, SSRs). They exhibit a high level of polymorphism, and like the RADP markers, they do not require primer sequence information (Gupta *et al.* 1994, Goodwin *et al.* 1997). ISSRs have been found to be suitable for determining intra- and inter-specific diversity since they reveal variation at several loci within the genome simultaneously (Zietkiewicz *et al.* 1994). The segregation of ISSR polymorphic bands is consistent with Mendelian inheritance [dominant (Tsumura *et al.* 1996) or codominant (Wu *et al.* 1994)].

ISSRs can also be easily used as a mapping tool (Sanker and Moore 2001). Akagi *et al.* (1996) produced an ISSR marker that was closely linked to the nuclear restorer gene (*Rf-1*)

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in rice. Disease resistance has also been successfully tagged with ISSR markers (Ratnaparkhe *et al.* 1998). A study of *Ipomoea* had previously shown that ISSRs yield considerable polymorphism (Huang and Sun 2000), which implies that an ISSR marker system may play an important role in the genetic analysis of sweetpotato.

In the present study, we examined the generation and detection of ISSR markers in sweetpotato, and evaluated the genetic relationships among different sweetpotato cultivars and related wild species using this DNA marker system. We also analyzed the mode of inheritance, segregation and linkage of ISSR markers.

Materials and Methods

Plant materials

The plant materials used in this study were divided into two groups.

The first group (Table 1) consisted of 28 *I. batatas* breeding lines or cultivars and 4 wild species (6 accessions) belonging to Series Batatas based on the classification of the International Potato Center (Huamán 1999). This group was used for phylogenetic and fingerprinting analysis.

The second group was a pseudo-test cross mapping population consisting of two parents 'Indonesia 47' and 'Kokei 14' and 87 F_1 individuals. The inheritance, segregation and linkage of ISSR markers were determined with this group.

DNA isolation

DNA was isolated from 1–2 g of young leaves following the CTAB method described by Doyle and Doyle (1990) with some modifications. Briefly, the powder was dispersed in 10 ml of extraction buffer (0.2 M EDTA, 0.1 M Tris pH 8.0, 1.4 M NaCl, 2% CTAB, plus 1.5% beta-mercaptoethanol added just before use) and incubated at 60°C for 1 hr. An equal volume of chloroform : isoamyl alcohol (24 : 1) was added. Following centrifugation, DNA was precipitated by isopropanol, rinsed with 70% ethanol, and then dissolved in 800 µl TE containing 20 µg RNaseA. After incubation at 37°C for 20 min, the DNA samples were stored at –20°C until use.

PCR analysis

A set of 100 primers (UBC Primer set no. 9) was obtained from the University of British Columbia, Canada. The reaction conditions were optimized to improve the banding patterns for each primer. The total reaction volume for amplification was 20 µl. Reaction mixtures contained 20 ng DNA, 0.2 µM of each primer, 200 µM of each dNTP, and 1 U GeneTaq (Nippon Gene) in 1 × PCR universal buffer (Nippon Gene). PCR amplifications were performed in a thermal cycler (MJ Research PTC-200 Peltier, USA) for 35 cycles after initial denaturation at 94°C for 5 min. Each cycle consisted of 45 s at 94°C, 45 s at 50–55°C (depending on the primers used), and 90 s at 72°C. A final extension was performed at 72°C for 5 min. To improve the quality of the banding patterns, 2% formamide was added to some of the reactions. PCR products were electrophoresed on 1.5% agarose gel or 6% polyacrylamide gel. λ/*Hind* III and/or pGEM/*Rsa* I were used as molecular weight markers to determine the size of the amplified fragments. All of the patterns generated were repeated three times to obtain reproducible data.

Table 1. Name and origin of sweetpotato and related wild species

Code	Species	Cultivar/accession name	Origin
1	*I. batatas*	Beniazuma	Japan
2	*I. batatas*	Benikomachi	Japan
3	*I. batatas*	Harukogane	Japan
4	*I. batatas*	Kankei 103	Japan
5	*I. batatas*	Kankei 105	Japan
6	*I. batatas*	Kankei 106	Japan
7	*I. batatas*	Kankei 109	Japan
8	*I. batatas*	Kankei 111	Japan
9	*I. batatas*	Kankei 107	Japan
10	*I. batatas*	Kankei 108	Japan
11	*I. batatas*	Kanto 115	Japan
12	*I. batatas*	Kanto 116	Japan
13	*I. batatas*	Koganesengan	Japan
14	*I. batatas*	Kokei 14	Japan
15	*I. batatas*	Kyukei 61	Japan
16	*I. batatas*	Kyukei 116	Japan
17	*I. batatas*	Kyushu 127	Japan
18	*I. batatas*	Tanegashimamurasaki	Japan
19	*I. batatas*	PH-3	Philippines
20	*I. batatas*	PH-5	Philippines
21	*I. batatas*	PH-6	Philippines
22	*I. batatas*	PH-8	Philippines
23	*I. batatas*	PH-SG	Philippines
24	*I. batatas*	94PH-12	Philippines
25	*I. batatas*	94PH-13	Philippines
26	*I. batatas*	94PH-14	Philippines
27	*I. batatas*	90SR-26	Malaysia
28	*I. batatas*	Indonesia 47	Indonesia
29	*I. lacunosa*	8059	Colombia
30	*I. tiliacea*	K270	Mexico
31	*I. trifida*	8036	Colombia
32	*I. trifida*	8048	Colombia
33	*I. triloba*	8066	Ecuador
34	*I. triloba*	8077	Mexico

Relationship analysis

The amplified fragments were scored manually for the presence or absence of unambiguous bands. Nei's genetic distance (1978) was calculated using the program TFPGA (Miller 1997) and a dendrogram of this genetic distance was constructed using Unweighted Pair-Group Method with Arithmetical Averages (UPGMA).

ISSR segregation assays

ISSR markers were scored for the absence or presence of reproducible bands. Markers present in one parent but absent in the other were chosen for inheritance analysis. Segregating markers were analyzed by a chi-square test for the

goodness of fit to an expected phenotypic segregation ratio. Linkage analyses were performed for simplex markers (1 : 1 segregation ratio, assuming chromosome segregation). Recombination fractions were determined using the maximum likelihood estimator for coupling, $r_c = (b + c)/(a + b + c + d)$, where a, b, c and d are the four classes of progeny AB, Ab, aB and ab (Wu *et al.* 1992).

Results

PCR amplification and preference in SSR sequence type

After screening of 100 primers (91 of which contained SSRs), 24 SSR primers were found to amplify scorable and reproducible banding profiles. The other primers were discarded since either they did not produce bands or there was a smear in the PCR products. The optimum conditions and the sequences of the primers are shown in Table 2. These 24 primers included 19 anchored and 5 unanchored primers. The 19 anchored primers included 16 microsatellite primers anchored at the 3′-end, while the other primers were anchored at the 5′-end. Based on the repeat motif length, the 24 primers were divided into 4 groups: 19 dinucleotide, 2 trinucleotide, 2 tetranucleotide and 1 pentanucleotide primers. Among the 19 dinucleotide primers, 10 consisted of GA and one CT, while four consisted of CA and four GT dinucleotide repeats. Dinucleotide SSR primers consisting of the bases A and T did not give any fragment amplification. Moreover, among the 3′-end-anchored dinucleotide SSR primers, 11 SSR primers containing (GA + CT) could amplify 65 scorable bands while 5 SSR primers containing (CA + GT) amplified 22 bands for cv. Kokei 14. Comparison of the PCR products obtained using the 8 SSR primers containing (CA + GT) showed that optimized 5′-anchored primers and 3′-anchored primers amplified similarly in terms of band number and size range.

ISSR polymorphism and genetic relationships

The PCR products generated from ISSR analysis were used to determine the genetic distances between sweetpotato and its related wild species. Eight primers were selected for their stable reproducibility and high polymorphism in DNA amplification patterns (Table 3). A total of 81 polymorphic loci were scored for the 8 SSR primers in the 34 accessions of sweetpotato and its related wild species. The number of polymorphic loci ranged from 7 to 13 per optimized primer, with an average of 10 polymorphic loci, and the PCR products ranged in size from 200 bp to 2,500 bp. Huang and Sun (2000) reported a higher level of polymorphism, which can be explained by the large number of *Ipomoea* species used in their analysis. The ISSRs generated were used to calculate the genetic distances between 34 accessions, and their relationships were revealed by a dendrogram (Fig. 1). The 34 accessions tested in this study were clustered into three major groups. The first group included 28 sweetpotato cultivars,

Table 2. Optimized PCR conditions for ISSR markers

Primer	Sequence	GC content (%)	Ta[1)]	Formamide (2%)	Amplified bands for Kokei14	Polymorphic bands in mapping population
807	$(AG)_8T$	47.1	52	+	5	2
808	$(AG)_8C$	52.9	50	+	7	4
809	$(AG)_8G$	52.9	55	+	12	5
810	$(GA)_8T$	47.1	50	+	4	1
811	$(GA)_8C$	52.9	53	–	13	3
815	$(CT)_8G$	52.9	53	–	4	3
819	$(GT)_8A$	47.1	50	+	5	5
825	$(AC)_8T$	47.1	54	–	6	7
834	$(AG)_8YT$	50/44.4	53	+	5	1
835	$(AG)_8YC$	50/55.6	54	–	3	2
836	$(AG)_8YA$	50/44.4	53	–	10	6
840	$(GA)_8YT$	50/44.4	52	+	7	4
841	$(GA)_8YC$	50/55.6	52	–	5	4
848	$(CA)_8RG$	50/55.6	52	–	7	0
850	$(GT)_8YC$	50/55.6	50	–	3	1
857	$(AC)_8YG$	50/55.6	55	–	1	2
861	$(ACC)_6$	66.7	52	–	4	5
864	$(ATG)_6$	33.3	50	+	10	1
873	$(GACA)_4$	50	50	+	6	5
878	$(GGAT)_4$	50	54	+	4	2
880	$(GGAGA)_3$	60	55	–	6	1
889	$DBD(AC)_7$	41.2/47.1/52.9/58.8	50	+	3	0
890	$VHV(GT)_7$	41.2/47.1/52.9/58.8	50	+	4	4
891	$HVH(TG)_7$	41.2/47.1/52.9/58.8	50	+	8	2

R = A,G; Y = C,T; B = C,G,T; D = A,G,T; H = A,C,T; V = A,C,G.

[1)] Ta: Annealing temperature.

Table 3. Primer sequences and polymorphic bands amplified in relationship analysis

Primer	Primer sequence	Polymorphic bands
808	$(AG)_8C$	9
810	$(GA)_8T$	12
811	$(GA)_8G$	13
815	$(CT)_8G$	7
835	$(AG)_8YC$	11
864	$(ATG)_6$	10
873	$(GACA)_4$	11
878	$(GGAT)_4$	8
Total		81

which could be clearly separated into two clusters. All of the cultivars from the Philippines and Indonesia were clustered together, and the other cluster contained almost all the Japanese cultivars. The second group contained two *I. trifida* accessions. The third group consisted of three wild species, *I. triloba* (two accessions), *I. tiliacea* and *I. lacunosa*. The genetic distances revealed that *I. trifida* was the most closely related wild species to cultivated sweetpotato among the wild species studied.

ISSR marker segregation

The primers that showed polymorphisms between 'Indonesia 47' and 'Kokei 14' were used to determine the mode of segregation, inheritance and linkage of the ISSR markers. According to the theories about the inheritance of sweetpotato (Kumagai *et al.* 1990, Kriegner *et al.* 2002), different segregation ratios of markers can be observed in the F_1 population due to allele dosage. Based on the presence or absence of bands in each parent, simplex and duplex markers were scored in this study. Out of the 24 functional SSR primers, 22 amplified 70 DNA bands that showed polymorphisms between the two parents (Fig. 2). The observed segregation ratio for 39 ISSR markers from the female parent and 31 from the male parent are shown in Fig. 3. A segregation analysis resulted in the identification of 49 simplex and 11 duplex markers (Table 4). The first step in the linkage analysis of hexaploids should be to identify polymorphic

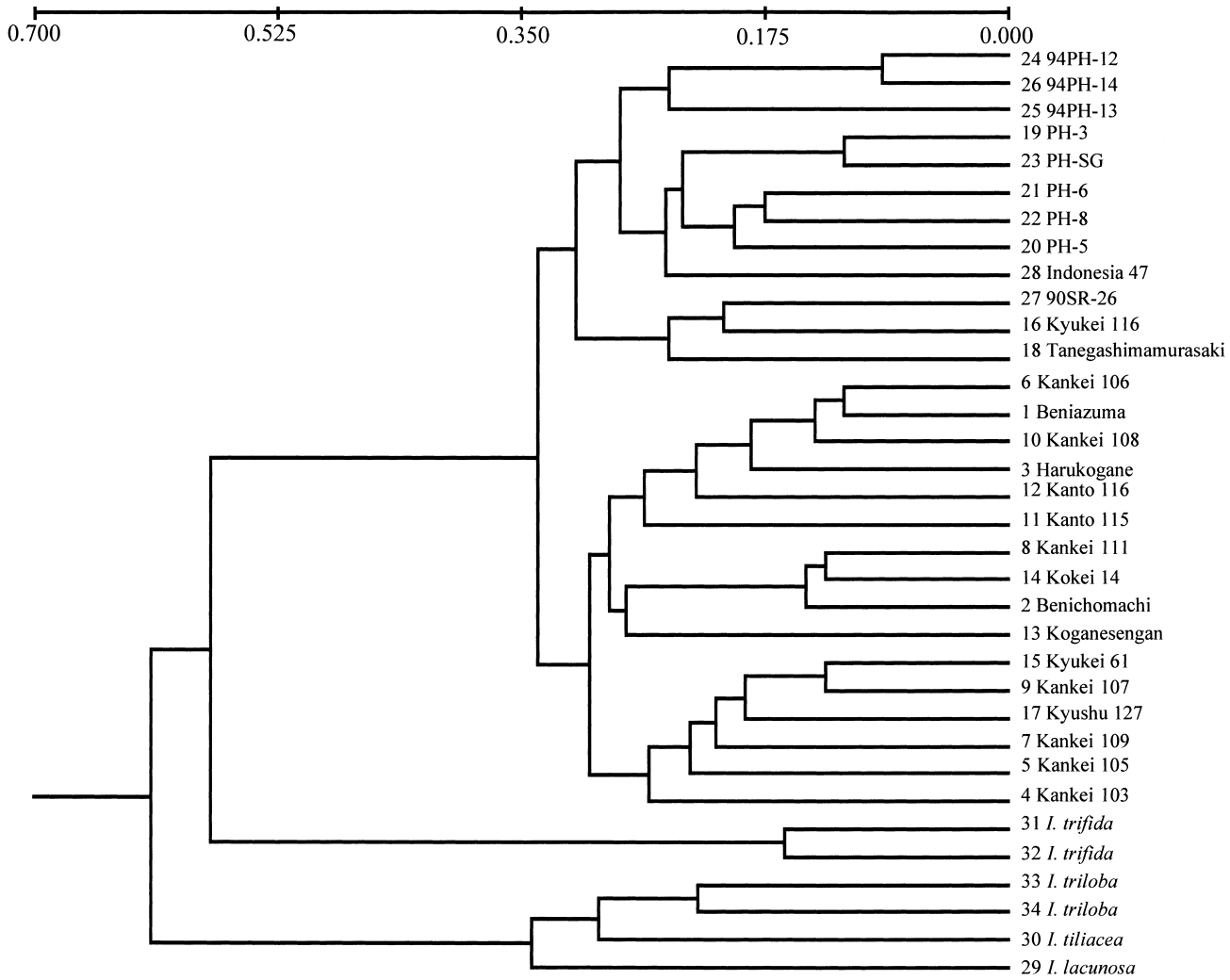

Fig. 1. Dendrogram of 34 accessions of sweetpotato and its related wild species generated by UPGMA cluster analysis with ISSR markers. The numerical scale indicates the genetic distance. The accession numbers correspond to those in Table 1.

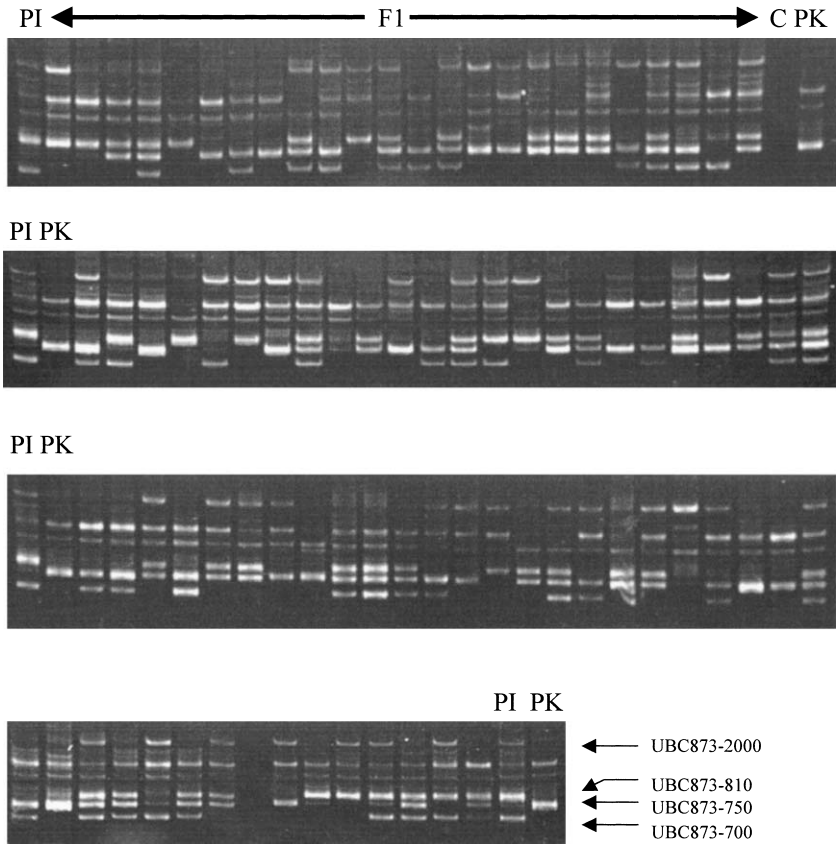

Fig. 2. Segregation of ISSR markers generated by UBC 873 in a pseudo-test cross of sweetpotato. PI: Indonesia 47, PK: Kokei 14, C: negative control. Other lanes: F_1 progeny. UBC873-2000, UBC873-810 and UBC873-700 are simplex markers; UBC873-750 is duplex marker.

markers in the coupling phase because a large number of progenies is needed to estimate repulsion linkages (Thompson *et al.* 1997). The 49 markers that segregated into 1 : 1 were included in two-point linkage analyses for each parent. The chi-square test ($\chi^2 = 39.8$, $P < 0.005$) of independence indicated that the ISSR markers UBC810-1600 and UBC873-700 were linked in the coupling phase for 'Indonesia 47' and the recombination fraction was 0.1839.

Discussion

PCR optimization and SSR trends in the sweetpotato genome

Optimum conditions were achieved for 24 SSR primers. Only a few primers produced optimal results under the same PCR conditions, even when the C + G content was similar or when the SSR composition was similar but with a different anchor. The addition of formamide improved the efficiency of PCR product formation with some primers, which indicated that formamide influences primer-template annealing and melting temperature (Tsumura *et al.* 1996, Huang and Sun 2000). The annealing temperature is a very important factor that affects the PCR amplification profile. Different primers require a specific annealing temperature, even when the theoretical annealing temperatures are calculated to be equivalent. Similar observations have been reported in other plants, such as rice (Blair *et al.* 1999) and citrus (Sanker and Moore 2001).

Although AT dinucleotide SSRs are the most abundant and are highly polymorphic in plant genomes (Wang *et al.* 1994, Akagi *et al.* 1997), the use of SSR primers consisting of the bases A and T makes it difficult to amplify specific bands. This finding was previously reported by Gupta *et al.* (1994) and Fang and Roose (1997). They suggested that the apparent lack of amplification observed with (A, T) dinucleotides may be due to self-complementarity within the primer. Studies have been so far directed toward the comparative efficiency of 5′- and 3′-anchored primers in PCR. It has been shown that 5′-anchored primers produced fewer but larger fragments with clearer patterns compared to those produced by 3′-anchored primers in *Citrus* (Fang and Roose 1997, Sanker and Moore 2001). The differences between 5′- and 3′-anchored primers may be a function of the number of bases present in the anchored sequence because most of the 5′-anchors tested contained three bases, while the 3′-anchors contained only one base. Sanker and Moore (2001) pointed out that the differences in the number of anchors contributed to the differences in the amplification. In this study, 5′-anchored primers and 3′-anchored primers generated a

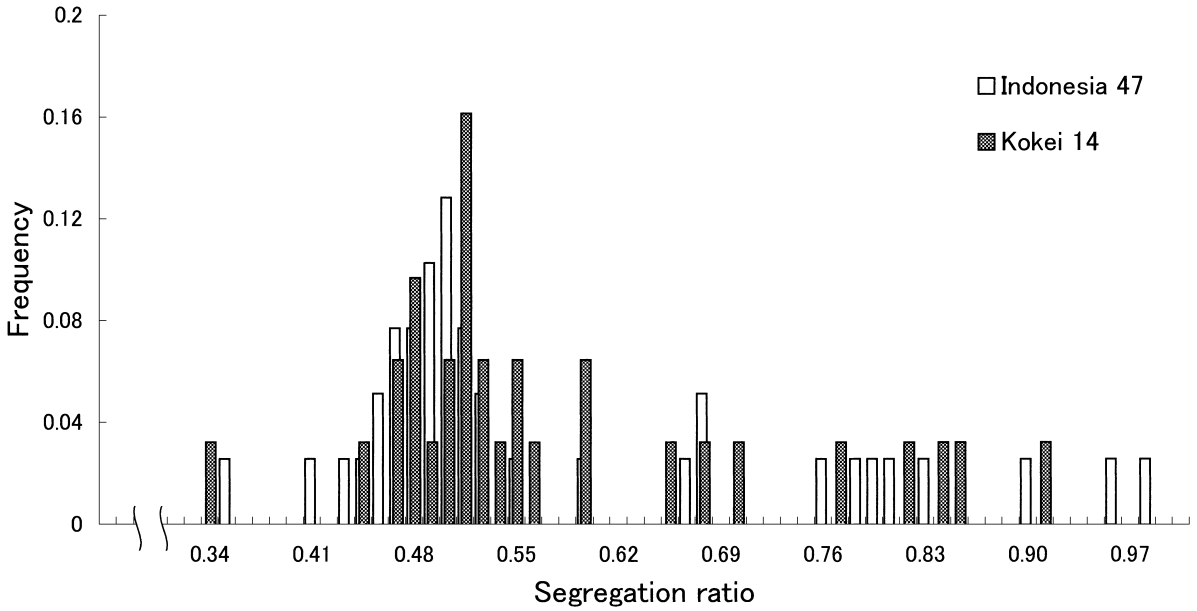

Fig. 3. Segregation ratio of ISSR markers for parental genotypes in 87 progenies. The white bars denote the frequency of 'Indonesia 47'-derived ISSR markers in the F_1 population and the gray bars denote the frequency of 'Kokei 14'-derived ISSR markers in the F_1 population.

Table 4. Segregation analysis of 70 ISSR markers in sweetpotato crossing between Indonesia 47 and Kokei 14

Marker type[3)]	Indonesia 47[1)]		Kokei 14[2)]		Total	
		%		%		%
Simplex	27	69.2	22	71.0	49	70.0
Duplex	6	15.4	5	16.1	11	15.7
Others	6	15.4	4	12.9	10	14.3
Total	39	100	31	100	70	100

[1)] Amplified PCR product present in Indonesia 47 but absent in Kokei 14.

[2)] Amplified PCR product present in Kokei 14 but absent in Indonesia 47.

[3)] Simplex and duplex markers are ISSR markers that segregated into a simplex pattern (1 : 1 ratio) and a duplex pattern (4 : 1 and 5 : 1), respectively. Others are ISSR markers that did not fit to the above ratios. Ratios were tested by a chi-square test ($P < 0.05$).

similar amplification pattern. However, since only CA/GT repeats were assessed in this experiment, our observations may only be valid for those repeat motifs. To elucidate the differences in the PCR amplification profile, it will be necessary to test a large number of 5′- and 3′-anchored primers with different repeat motifs.

If microsatellites were randomly distributed along the genome, the length of the intervening regions between simple sequence repeats of the same motif should be a function of their frequency and of the number of bands produced by SSR primers with a given microsatellite repeat that reflect the relative frequency of that motif (Blair *et al.* 1999). In our experiment, 11 SSR primers containing (GA+CT) amplified 65 bands, while 5 SSR primers containing (CA+GT) amplified 22 bands, suggesting that there are more GA or CT binding sites than CA or GT binding sites in the region that can be amplified. The finding that there were more GA dinucleotide SSRs than CA repeat SSRs is in agreement with the results of sweetpotato genomic library screening (data not shown). Trinucleotide, tetranucleotide and pentanucleotide SSR primers without an anchor were also successfully optimized in the present study. The optimization of these SSR primers with a long repeat motif suggests that SSRs with a long repeat motif are abundant along the sweetpotato genome. SSRs with a long repeat motif are more intriguing because they show a high polymorphism and are easy to score (Lu *et al.* 1996). ISSR information regarding the motif type, frequency and polymorphism of different SSR motifs would be useful for developing new microsatellite markers of sweetpotato, which could be suitable for constructing a linkage map.

Polymorphisms revealed by ISSR-PCR

ISSR techniques have been widely applied to assess genetic diversity in several economically important plants and are useful for biological conservation. To date, the classification of Series *Batatas* is not reliable because the morphological characteristics can vary considerably under different environmental conditions. Due to the high polymorphic levels of ISSR markers, genetic diversity and relationship analyses can be performed for sweetpotato and its related wild species (Huang and Sun 2000). The eight selected primers used in this study could distinguish all the 34 accessions from each other. The ISSR markers seem to be quite valuable for identifying sweetpotato cultivars and for evaluating their genetic diversity. Austin (1988) noted that *I. trifida* is one of the most likely ancestors of the cultivated sweetpotato.

The results of DNA marker analysis also confirmed that *I. trifida* is a diploid species that is closely related to the cultivated hexaploid *I. batatas*, while *I. tiliacea*, *I. ramosissima* and *I. umbraticola* are more distantly related to *I. batatas* (Jarret *et al.* 1992, Jarret and Austin 1994, Dhillon and Ishiki 1999, Huang and Sun 2000). Our results on all possible pairwise genetic distances calculated between accessions showed that *I. trifida* is closely related to cultivated sweetpotato, which supports the traditional view. Comparative studies of genetic relationships showed that ISSR markers could provide the same level of accuracy as RFLP markers and chloroplast DNA restriction analysis (Nagaoka and Ogihara 1997, Huang and Sun 2000). Our results also support the usefulness of ISSR for resolving the genetic relationships at both intra- and inter-specific levels compared to the results obtained with RFLP and RAPD markers (Jarret *et al.* 1992, Komaki *et al.* 1998, Dhillon and Ishiki 1999).

Inheritance, segregation and linkage of ISSR markers

A pseudo-test cross mapping population is suitable for species with self-incompatibility and a high level of heterozygosity. This strategy has been used to map RAPD markers and AFLP markers for sweetpotato (Ukoskit and Thompson 1997, Kriegner *et al.* 2002). Of the 70 ISSR markers generated in this test, 60 were simplex and duplex markers that could be used for linkage analysis in the parents. Ten ISSR markers showed non-simplex and non-duplex segregation patterns, 6 markers were derived from Indonesia 47, while 4 from Kokei 14. Non-simplex and non-duplex ISSR markers in the present pseudo-test cross population were slightly more prevalent (14.3%) than RAPD markers (3.6% and 4.1% triplex and distorted markers, respectively) in a previous attempt to construct a linkage map of sweetpotato (Ukoskit and Thompson 1997). The distorted loci could be attributed to the small number of markers surveyed or to the small size of the mapping population.

Many ISSR markers have been used to extend existing linkage maps in plants, and have been shown to be useful for the development of linkage maps (Sanker and Moore 2001). Only one pair of linked ISSR markers was found in the present study. The low number of linkages is probably due to the large chromosome number in sweetpotato. A large number of chromosomes decrease the probability of linkage among markers compared to other species with fewer chromosomes. The low number of linkages also implies that SSRs are distributed evenly in the sweetpotato genome. The segregation results show that the use of ISSR markers is one of the approaches for the genetic analysis of sweetpotato.

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