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Isolation and Characterization of Novel Microsatellite Markers in Chayote [*Sechium edule* (Jacq.) Sw.]

Ryoko Machida-Hirano[1], Moisés Cortés-Cruz[2], Blanca Amalia Amaro González[2], Jorge Cadena Íñiguez[3], Kazuto Shirata[1,4], Kazuo N. Watanabe[1*]

[1]Gene Research Center, University of Tsukuba, Tsukuba, Japan
[2]Centro Nacional de Recursos Genéticos, Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, Tepatitlán de Morelos, México
[3]Colegio de Postgraduados, Campus San Luis Potosí, Salinas de Hidalgo, Mexico
[4]National Institute of Agrobiological Sciences, Tsukuba, Japan
Email: *nabechan@gene.tsukuba.ac.jp

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Abstract

Chayote [*Sechium edule* (Jacq.) Sw.] is an economically important species in Latin America; however, there are very few reports available regarding its genetic diversity. Out of 11 microsatellite markers isolated, 10 loci provided 1 to 7 alleles per locus in a set of Mexican chayote accessions. Observed and expected heterozygosities for each locus ranged from 0.00 to 0.85 and 0.00 to 0.73, respectively. The overall genetic diversity detected by microsatellites was compared with that detected by P450-based analogue markers, a genome-wide dominant marker. Genetic diversity values obtained by the newly designed microsatellite markers were almost equal to the value estimated by PBA markers, but genetic distances calculated by both marker systems were not significantly correlated. Additional microsatellite markers, which could detect more polymorphisms, may be necessary to analyze the genetic diversity and structure of Mexican chayote collections.

Keywords

Chayote, SSR Markers, PBA Markers, Dual-Suppression-PCR Technique, Genetic Diversity

1. Introduction

Chayote [*Sechium edule* (Jacq.) Sw.] is an economically important species of Cucurbitaceae in Latin America;

*Corresponding author.

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however, very few reports are available regarding its genetic diversity. Commercial production for local consumption and export of the fresh fruit is a significant source of revenue and local employment in Mexico [1]-[4] The most popular variety, "Verde Liso" (Smooth Green), has exported to international market and created local employments [1] [5]. In addition, it is also an important staple food for low-income groups in Latin America and is commonly grown in home gardens for family consumption [6]. In addition to consuming the fruits as vegetable, the tuberous roots are rich sources of starch and the shoots and young leaves are cooked as pot-herb [7].

Historical records and linguistics, occurrence of wild forms, and the distribution of related wild species indicate that the geographical origin is in tropical America, especially in Mexico and Central America [7]-[9]. In Mexico, the highest levels of chayote diversity exist in the states of Veracruz, Chiapas, and Oaxaca, especially in the central region of Veracruz where significant variation in fruit size, form and color, flavor, and skin texture (smooth or with spines) has been reported [10].

Although morphological variation has been reported [9] [10], studies on genetic diversity of chayote have been limited to the use of isozymes [11] [12] and AFLP [13]. Hyper variable molecular markers, such as microsatellites, offer higher resolving power for population genetics and are widely used to study genetic diversity, population structure, and genetic relatedness [14]. However, such tools are not available for chayote despite its economic importance. Therefore, development of species-specific molecular markers is needed to elucidate the genetic structure of the species.

In this paper, first we report the design of 11 species-specific microsatellite markers using Japanese chayote varieties. Second, the designed markers were evaluated by using 20 Mexican chayote accessions. Finally, results of genetic diversity analysis with the newly designed microsatellite markers were compared with those detected by P450-based analogues (PBAs) markers [15], which had been used to assess genome-wide diversity of P450-related functional genes on different plant species [16]-[19].

2. Materials and Methods

2.1. Microsatellite Marker Design and Evaluation

Total genomic DNA was extracted from dried leaves of the Japanese local variety of chayote, "Zairai Wase", by CTAB method [20]. Microsatellites were developed according to a previously described dual-suppression-PCR technique [21]. First, DNA was digested with *Alu* I, *Eco* RV, *Hae* III, and *Ssp* I restriction enzymes to construct four DNA libraries. The restricted fragments were then ligated by using a DNA Ligation Kit Ver.2.1 (Takara Bio, Japan) with specific blunt adaptors (the first consisted of a 48-mer: 5'-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3' and the second consisted of an 8-mer, with the 3'-end capped by an amino residue: 5'-ACCAGCCC-NH_2-3').

Flanked regions of the microsatellite were isolated from these four DNA libraries by amplification using compound microsatellite primers $(AC)_{10}$ or $(GA)_{10}$ and the adaptor primer AP2 (5'-CTATAGGGCACGCGTGGT-3') designed from the 48-mer adapter. The amplified fragments were then integrated into a plasmid pGEM-T vector using pGEM-T Easy Kit (Promega); the plasmids were then transferred into *Escherichia coli* DH5*α*, according to the manufacturer's instructions. The cloned fragments were amplified from the extracted plasmid DNA of positive clones using M13 forward and M13 reverse primers. The size of inserted fragments were checked by 1% agarose gel electrophoresis and the PCR products were directly sequenced with the M13(-21) primer using BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technology) in the ABI 3130 Genetic Analyzer (Life Technology). Each fragment containing $(AC)_n$ or $(GA)_n$ sequence at one end was chosen for the next step.

The next step consisted of determining the sequence of the other flanking regions for each microsatellite. A pair of primers, IP1 and IP2, was designed from each sequence. IP1 was a nested specific primer, designed from the flanking region of the microsatellites, whereas IP2 was designed based on the sequence between IP1 and the microsatellite. All these primers were designed using Primer3Plus [22]. An adapter-primer for nested PCR (AP1: 5'-CCATCGTAATACGACTCACTATAGGGC-3', designed from the 48-mer adaptor sequence) was also prepared. Primary PCR reactions were conducted on each constructed DNA library using the combination of IP1 and AP1 primers. Secondary PCR was conducted with a 100-fold dilution of the primary PCR products with IP2 and AP2. Single-banded fragments were then sub-cloned and sequenced. Finally, the primers IP3-forward and IP3-reverse were designed for each locus from the newly identified sequence between the AP2 and microsatellite sequences. The primer pairs IP3-forward/IP3-reverse were defined as the newly designed microsatellite markers.

Amplification of the newly designed markers was tested on 20 Mexican chayote accessions comprising 10 varietal groups with different fruit morphological characteristics (**Supplementary Table 1**). To obtain fluorescent-labeled products, amplification reactions were carried out using a 10 µl three-primer combination [23] as follows: 10 ng of genomic DNA; 5 µl of RedTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich); and 0.2 µM of fluorescent-labeled universal primer, 1.5 µM of locus-specific forward primer with 5' universal tails sequence, and 0.5 µM of locus specific reverse primer. PCR products were amplified using the following parameters: initial denaturation at 95˚C for 3 min; followed by 30 cycles of 95˚C for 30 s, 60˚C for 30 s, and 72˚C for 1 min; and a final elongation step at 72˚C for 1 h. The size of the PCR products was determined by capillary sequencer (3500 ×l Genetic Analyzer, Life Technologies).

General genetic diversity values were calculated at each locus. Pair-wise genetic distances among 20 individuals were calculated using "Codom-Genotypic" option to enable comparisons between codominant (microsatellites) and dominant (PBA) data output. Principal coordinate analysis (PCoA) was also carried out. All these analyses were carried out using GenAlEx 6.5 [24]. Polymorphism information content (PIC) of each marker was calculated using a PIC calculator [25]. The characteristics of the microsatellite markers for Mexican chayote are shown in **Table 1**.

Table 1. Characteristics of 11 microsatellite loci in *Sechium edule*. The observed and expected heterozygosity were calculated for the 20 individuals of Mexican chayote.

Locus		Primer sequence 5' > 3'	Repeat motif	Genbank no.	Size[a] (bp)	N	Na	*Ho*	*He*	PIC
Sed 01	F:	CCCCGTTACCCTGACTCTCGAT	$(CA)_8$	AB871395	196	20	2	0.85	0.50	0.37
	R:	GGCTTGTTCAAGACTTCGCAGC								
Sed 02	F:	AGAAGACGACACACTTTTGAGCA	$(CA)_2C\ (CA)_5$	AB871396	316	20	1	0.00	0.00	0.00
	R:	ATCTACCCGTGACTGCCCAGAT								
Sed 03	F:	CGTATGGTCGAGGTGCGCATAA	$(CA)_9$	AB871397	144	20	5	0.15	0.51	0.48
	R:	AAGTCCAGAAATGTACACTGCCACT								
Sed 04	F:	GGCCCTTAGTTTGCTGATGGGT	$(CA)_2CT(CA)_2CC(CA)_3$	AB871398	378	20	1	0.00	0.00	0.00
	R:	TGGGACCCACGTGCTAAAAGTG								
Sed 05	F:	ACACACCTTAGAAAGAGCAACCCC	$(CA)_2CGA(CA)_5$	AB871399	274	20	1	0.00	0.00	0.00
	R:	GCTATGGCGCAAGTTGCTGATG								
Sed 06	F:	AACCGCTGTTCTCTGCTCATCC	$(CA)_4TA(CA)_{16}$	AB871400	229	20	3	0.15	0.14	0.14
	R:	GGCTCAAGGTTGTTGTTGGTGC								
Sed 07	F:	AACCTGGGTCGTTACATGGTGC	$(GA)_{34}$	AB871401	359	20	3	0.15	0.22	0.21
	R:	ACCCTTGCCCTAGATGGTGGAA								
Sed 08	F:	AGCTCCTCCACCTCTACCTTTTGC	$(GA)_3GC(GA)_5$	AB871402	382	20	3	0.15	0.14	0.14
	R:	ACTCTGGCGTATGGAATGACGC								
Sed 09	F:	ACAGGCCACAGGGGAACAAAAT	$(GA)_{11}$	AB871403	204	20	5	0.20	0.57	0.51
	R:	CACGCCATCTCCGTCCATCTTT								
Sed 11	F:	TGGTCTGTTTGGCTCATCTCCA	$(GA)_{14}$	AB871405	299	20	7	0.11	0.73	0.68
	R:	TCGACCCCTAACCCTTGAAGCT								
Mean						20	3.1	0.21	0.28	0.25

[a]PCR product with Japanese variety "Zairai wase". Note: N = sample size; Na = Number of effective alleles; *Ho* = observed heterozygosity; *He* = expected heterozygosity; PIC = polymorphism information content.

2.2. Genetic Diversity Analysis of PBA Marker

Genetic diversity values from the same set of Mexican chayote samples were evaluated using PBA markers [11] [15]. The PBA primer sets comprised of three forward primers (CYP1A1F, CYP2B6F, and CYP2C19F) and three reverse primers (CYP1A1R, CYP2B6R, and CYP2C19R). Nine combinations of these primer pairs were tested and eight of them produced scorable bands (**Table 2**). PCR amplifications were performed using 10 ng of template DNA in a total reaction mixture of 10 µl containing 5 µl of RedTaq® ReadyMix™ (Sigma-Aldrich) and 0.18 µM each of forward and reverse primers. This PCR was run at 94˚C for 5 min, followed by 30 cycles of 94˚C for 1 min, annealing temperature for 2 min, and 72˚C for 3 min, with a final extension of 72˚C for 10 min. Annealing temperature varied for each primer set, as shown in **Table 2**. PCR products were electrophoresed using 6% of acrylamide gels at 150 V for 3 h, followed by silver staining.

For statistical analysis, amplified DNA fragments were scored in a binary data matrix, where the presence of band was denoted as 1 and its absence as 0. A single data matrix was prepared by combining genotyping results of 8 primer combinations and general genetic diversity values were calculated. Genetic distances among individuals were calculated and PCoA was also carried out. The correlation between estimates of genetic distances based on PBA and microsatellite data was determined by Mantel test [26]. All data analyses were carried out using GenAlEx 6.5 [24].

3. Results and Discussions

3.1. Design of Microsatellite Markers for Chayote and Genetic Diversity Values of Mexican Chayote

A total of 40 clones from the $(AC)_{10}$ and 24 clones from the $(GA)_{10}$ libraries were randomly chosen based on amplification size (≥500 bp) and were sequenced. Eighteen (45%) and 9 (37.5%) clones, respectively, from each library, had enough sequence length to design two primers between repeat motif and adapter sequences, and they were used to design IP1 and IP2 primers. In total, 15 primer pairs were designed, 7 from the $(AC)_{10}$ library and 8 from the $(GA)_{10}$ library. These primers were initially tested on a subset of *S. edule* individuals from the Japanese varieties to confirm reliable amplification. Of these, 4 amplified multiple loci while 11 were polymorphic and produced a single band (GenBank accession no.: AB871395 to AB871405).

Specie-specificity of the newly designed markers was confirmed by cross amplification with arbitrary selected other plant species. Four genotypes of heliconias (*Heliconia bourgaeana*, *H. bihai*, *H. collinsiana* and *H. uxpanapensis*) and four chayote genotypes were tested with primers designed in this study. Amplifications were observed only from chayote DNA samples, and not from heliconias. Equally, cross-amplification of microsatellite markers designed for heliconias (Hac-A116 and Hac-D1) [27] were tested with chayote samples. PCR products were observed only in the four *Heliconia* spp. DNA samples (**Supplemental Figure 1**). DNA sequences from the selected libraries were used to do a homology search against NCBI Nucleotide collection database (http://www.ncbi.nlm.nih.gov/), using blastn and no significantly high homology sequences were found (data not shown), confirming that DNA from chayote was used during the primers design and that DNA cross contamination with other species was avoided.

Table 2. Combinations of PBA markers used in this study.

Primer pairs/combinations	Annealing temp. (˚C)	Number of polymorphic bands
CYP1A1F/CYP1A1R	56	15
CYP1A1F/CYP2B6R	52	11
CYP1A1F/CYP2C19R	47	9
CYP2B6F/CYP1A1R	52	5
CYP2B6F/CYP2C19R	47	14
CYP2B6F/CYP2B6R	52	15
CYP2C19F/CYP1A1R	56	17
CYP2C19F/CYP2C19R	47	2
Average	-	11

The 11 polymorphic primer pairs were further tested on 20 individuals from a Mexican collection (BANGESe, National *Sechium edule* Germplasm Bank, Huatusco, Veracruz, Mexico). One primer combination (*Sed*10) was discarded from further analysis because it produced too much stutter bands and size determination was not possible. Out of 10 loci tested, 7 microsatellites showed polymorphism, ranging from 1 to 7 alleles per locus (**Table 1**). The mean values for effective allele number, observed heterozygosity (*Ho*), and expected heterozygosity (*He*) were 3.1, 0.21, and 0.28, respectively. Polymorphism information values ranged from 0.00 to 0.68. The proportion of polymorphic loci was 70%, whereas isozyme studies reported 59.8% [5] and 57.14% [11], suggesting that microsatellite markers had higher resolution in detecting polymorphisms within chayote varieties.

Among 20 Mexican chayote individuals tested, only one pair (SE-369 and SE-288) demonstrated identical patterns of allelic combinations, both of them belonged to the same varietal group, *albus levis*, and share identical fruit characteristics. However, individuals that belonged to other varietal groups (e.g., *albus minor*, SE-261 and SE-330) exhibited different genotyping patterns. There is still a need for development of additional markers to increase resolution so that microsatellite markers can be used for genotype identification and, in some cases, for marker-assisted selection. However, these results suggest that the DNA profiles based on the application of newly designed microsatellite markers could be used to differentiate chayote individuals and accessions.

3.2. Low Heterozygosity Detected in Chayote Collection

Chayote is known as a predominantly cross-pollinated species but its low heterozygosity is reported by isozyme studies [1] [11]. In the present study, 9 out of 10 newly designed microsatellite markers also detected low level of heterozygosity (*Ho* = 0.00 to 0.20), except for *Sed* 01 (*Ho* = 0.85). Other genetic diversity studies on Cucurbitaceae using microsatellite markers demonstrated moderate to high heterozygosity (0.49 - 0.75 [28], 0.26 - 0.79 [29], 0.00 - 1.00 [30], and 0.325 - 0.867 [31]). Although individual species in the Cucurbitaceae family are generally cross-pollinated, chayote is known to be self-compatible [32] and a single plant of chayote shows a good fruit set and does not demonstrate clear inbreeding symptoms [33]. Therefore, cross-pollination rate in cultivated chayote may not be as high as assumed, since chayote is typically cultivated in home gardens and a small number of plants are used for seed source [11]. Reference [34] also reported low heterozygosity values (range 0.00 - 0.50) in melon samples evaluated with a set of 25 microsatellites and mentioned possible favoring endogamy in cultivated populations. Pollination with self and/or among close relatives during domestication and cultivation of the species may have contributed to the observed low heterozygosity values.

3.3. Relationship between Morphological Characters and Genetic Marker Grouping Patterns

Although the set of Mexican chayote collection consisted of a broad range of varietal group with high diversity in fruit morphology (**Supplementary Table 1**), the scatter diagram of the PCoA derived by both microsatellite and PBA markers did not show a clear distribution pattern associated with a varietal group or fruit morphological characteristics (data not shown). Fruit morphological characteristics, such as color, shape, size, taste, and presence/absence of spines, are not environmental adaptation-related traits, unless directional selections by humans have strongly influenced chayote domestication. In addition, [35] reported that spines on fruits can be controlled by gibberellic acid application during development. Therefore, a clear relationship between genetic and morphological groupings may not have been observed.

3.4. Comparison of Microsatellites and PBA Marker Systems in Detecting Genetic Diversity

From the 9 primer combinations evaluated for the PBA markers, 8 showed polymorphisms on the same set of Mexican chayote samples. The total number of polymorphic bands was 88, ranging from 2 (CYP2C19F/CYP2C19R) to 17 (CYP2C19F/CYP1A1R) (**Table 2**). The mean *He* value calculated with the PBA marker system was almost the same as that obtained by the microsatellite marker system (0.29 vs. 0.28, respectively), indicating that the 10 newly designed microsatellite markers in this study were useful in detecting genetic diversity levels of a Mexican chayote collection set. Mantel test showed that there was no significant correlation between the respective genetic distances estimated by the microsatellite and PBA data ($r = -0.051$; $p = 0.40$). This may be due to the existence of a genome-wide distribution of P450-related functional genes, which

are detected by PBA markers, whereas microsatellites are detected polymorphisms at a single locus. These results highlight the different properties of each marker system. In a multivariate analysis by using microsatellite and PBA markers in rice, it also revealed different distribution patterns, and mentioned that the different types of molecular markers might contribute in the different aspects of the genetic structure [19]. Therefore, evaluation of genetic diversity combining different assessment methods would be helpful, especially for the species lack number of species specific markers available.

4. Conclusion

Microsatellite markers are widely used for fingerprinting and diversity studies of different species. In this study, we reported the design of 11 microsatellite markers for chayote. Ten of them were easily genotyped and 7 of them detected polymorphisms in a set of Mexican chayote accessions. When compared with the results obtained by 8 combinations of PBA markers, the genetic diversity indicators calculated by these microsatellites were almost equivalent but demonstrated different grouping patterns. However, the designed markers sets were not efficient enough to discriminate clearly different genotypes. It may be necessary to develop more microsatellite markers in order to detect more polymorphisms, more precise genotyping, and larger scale genetic diversity germplasm collections. Currently, development of additional microsatellite markers is under process and these markers will be useful to characterize population genetic structures of chayote varieties of Mexico and Latin America and to decipher the dynamics of its genetic diversity.

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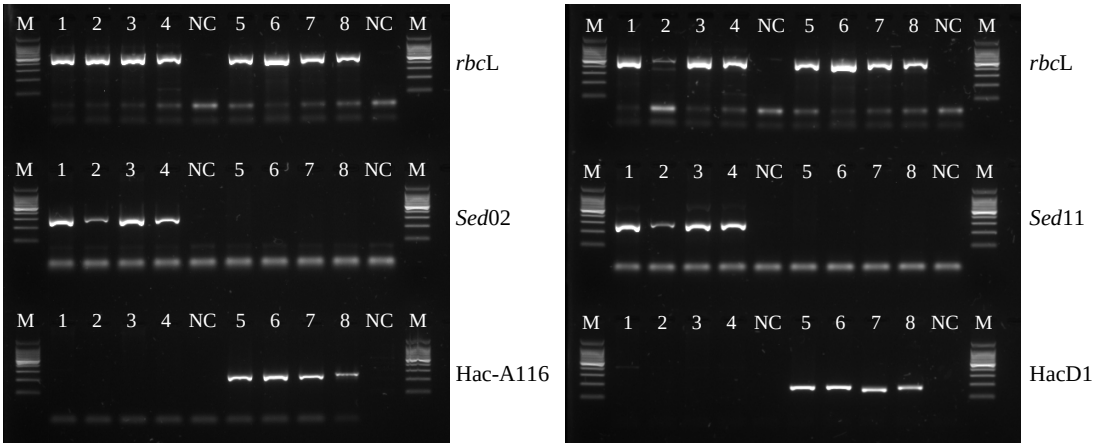

Supplementary Figure 1. Specificity of two designed microsatellite markers (*Sed* 02 and *Sed* 11) for chayote with DNA of other plant species (*Heliconia acuninata*). (a) PCR amplification with *rbc*L (positive control), *Sed* 02 (species specific markers to *Sechium edule*), and Hac-A116 (species specific marker of *H. acuminata*); (b) PCR amplification with *rbc*L (positive control), *Sed* 11 (species specific markers to *S. edule*), and Hac-D1 (species specific marker of *H. acuminata*). M, molecular weight marker (100 bp DNA ladder); 1 - 4, four distinct genotypes of *S. edule*; 5 - 8, *Heliconia* sp.; NC, negative control (without DNA).

Supplementary Table 1. List of Mexican chayote (*Sechium edule* (Jaqcs.) Sw.) used in this study with distinctive fruit characteristics.

Accession no.	Variety group name	Fruit color	Fruit shape	Fruit size[a]	Taste	Spines
SE-261	*albus minor*	yellow	round	small	sweet	no
SE-264	*nigrum levis*	dark green	obovoid	medium	neutral	no
SE-264(b)	*nigrum levis*	dark green	obovoid	medium	neutral	no
SE-275	*albus dulcis*	yellow	pyriform	small	sweet	no
SE-277	*nigrum spinosum*	dark green	pyriform	big	neutral	yes
SE-284	*albus spinosum*	yellow	pyriform	big	sweet	yes
SE-286	*albus dulcis*	yellow	pyriform	small	sweet	no
SE-288	*albus levis*	yellow	obovoid	medium	sweet	no
SE-290	*virens levis*	dark green	obovoid	medium	neutral	no
SE-292	*nigrum levis*	dark green	obovoid	medium	neutral	no
SE-300	*nigrum spinosum*	dark green	pyriform	big	neutral	yes
SE-301	*virens levis*	dark green	pyriform	medium	neutral	no
SE-316	*nigrum spinosum*	dark green	pyriform	big	neutral	yes
SE-330	*albus minor*	Yellow	round	small	sweet	no
SE-331	*nigrum conus*	dark green	conical	small	neutral	no
SE-339	*nigrum xalapensis*	dark green	pyriform	big	neutral	no
SE-349[b]	*nigrum minor*	dark green	pyriform	small	neutral	no
SE-349[b]	*nigrum minor*	dark green	round	small	neutral	no
SE-354	*nigrum spinosum*	light green	pyriform	medium	neutral	yes
SE-369	*albus levis*	Yellow	obovoid	medium	sweet	no

[a]big (10.5 - 15 cm), medium (5.7 - 8.2 cm), small (>3 cm); [b]Both accessions share the same accession number but the fruit shapes are different.