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BRIEF COMMUNICATION

Development of novel chloroplast microsatellite markers for *Cucumis* from sequence database

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Abstract

The development of chloroplast microsatellite (cpSSR) markers in *Cucumis* species and analysis of their polymorphism and transferability were reported. Fifteen microsatellite markers, represented by mononucleotide repeats, were developed from the complete sequence of *Cucumis sativus* chloroplast genome. Intraspecific variation was successfully detected in *C. sativus* and *C. melo* and revealed mean 1.6 and 1.9 alleles per cpSSR locus, respectively. With the exception of two exon region-located cpSSR markers being monomorphic, each of the others amplified polymorphic fragments in *C. sativus* or *C. melo*. A total of 34 polymorphic loci were detected with these cpSSR markers in the two species. Transferability of the newly developed cpSSR markers was checked on an additional set of 41 *Cucurbitaceae* accessions (belonging to 12 different species), and except for two markers with no amplification in *Cucurbita maxima*, the others could be transferable to all the accessions tested. Of the 15 cpSSR markers, 14 markers generated fragments with expected band sizes and 13 markers detected interspecific polymorphism among the accessions. Intraspecific polymorphism was also observed within four *Cucurbitaceae* species excluding *C. sativus* and *C. melo*.

Additional key words: *Cucurbitaceae*; monomorphism, polymorphism; transferability.

Microsatellites or simple sequence repeats (SSRs) are widely distributed throughout nuclear and cytoplasmic genome in eukaryotes. Compared to nuclear genome, organelle genome is typically non-recombinant, uniparentally inherited and effectively haploid (Birky 1988). In plant, chloroplast genome is characterized by the small size, high copy number and relatively conserved gene order (Provan *et al*. 2001), which makes possible to detect relatively rapidly mutating sites in chloroplast genome by chloroplast microsatellite (cpSSR) marker. In most instances, chloroplast variation detected by cpSSR marker was much higher than by restriction fragment length polymorphism (RFLP) (Provan *et al*. 1997) and this offers convenience for the analysis of inter- and intra-specific chloroplast variation within plant population(s) by simple PCR techniques. Recently, cpSSR markers have been developed for a variety of plant species and proved to be invaluable in studies of plant population structure, diversity, differentiation and maternity analysis.

The genus *Cucumis* contains two major crop species, *Cucumis sativus* and *C. melo*, each of which has several ecotypes spreading over the world. Because of their economic importance, a large number of nuclear SSR markers have been developed (Danin-Poleg *et al*. 2001, Kong *et al*. 2006, 2007, Watcharawongpaiboon and Chunwongse 2008). However, there is a lack of cpSSR markers capable of detection of high-level polymorphism in *Cucumis* and the other closely related genera. In addition, development of cpSSR markers and analysis of *Cucumis* plastome polymorphism allow a more efficient search for cytoplasm donors that can be used to breed new cultivars, especially for cucumber that has a narrow genetic base (Skálová *et al*. 2008). In present study, we developed a set of cpSSR markers for *Cucumis* and examined their transferability on the other *Cucurbitaceae* species.

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Abbreviations: SSR - microsatellite or simple sequence repeat; cpSSR - chloroplast microsatellite; RFLP - restriction fragment length polymorphism; CTAB - cetyltrimethylammonium bromide.
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The availability of the complete sequence of *C. sativus* chloroplast genome (GenBank no. DQ865975) makes possible to develop new cpSSR markers. Fifty two SSRs, containing poly(A/T)$_n$ of at least 10 bp in length, were identified from *C. sativus* chloroplast genome. The program *TANDEM REPEATS FINDER* was used to locate these repeats in the chloroplast-genome sequence. Primers were designed to flank the cpSSRs using the software *Primer Premier 5.0* (*PREMIER Biosoft International*), and allowed generation of PCR products 100 - 300 bp in length and annealing temperature of 50 - 60 °C (Table 1).

Thirty one cucumber inbred lines [including 16 North China ecotypes, 9 South China ecotypes, 5 European greenhouse ecotypes and one wild related species (*C. sativus* var. *xishuangbannanesis*)] and 39 melon inbred lines [including 21 thick-peel types, 17 thin-peel types and one wild species (*C. melo* var. *agrestis*)] were used to screen cpSSR variation in cucumber and melon, respectively. Additionally, *Cucurbitaceae* species represented by 41 inbred lines or cultivars (Table 2), were used to examine the transferability of the cpSSR markers and their intraspecific polymorphism. The materials of *C. sativus* and *C. melo* were collected from National Mid-term Genebank for Watermelon and Melon, Zhengzhou, China, and the others were supplied by Group of Plant Genetics and Breeding, Henan Agricultural University, China. Total genomic DNA was extracted from all the materials mentioned above (inbred lines or cultivars) by cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980).

PCR reaction was done in a volume of 0.015 cm^3 containing 1× PCR buffer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 0.2 μM of each primer, 0.4 unit of *Taq* DNA polymerase (*TaKaRa Inc*., Dalian, China) and 20 - 50 ng DNA template. All amplifications were carried out on a *UNO II Biometra* thermocycler with the following profiles: 5 min of denaturation at 94 °C followed by 30 cycles of 50 s of denaturation at 94 °C, 50 s of annealing at annealing temperature and 1 min of extension at 72 °C and a final extension step of 72 °C for 8 min, stored at 4 °C. The products were electrophoresed on 6 % denaturing polyacrylamide gel and each gel included two lanes of Marker 11 (*pUC19/MspI*, *Nippon Gene*, Tokyo, Japan). The gels were silver-stained according to the technical

Table 1. The details of chloroplast microsatellite markers and their variations in *Cucumis*. Location, repeat motifs, primer sequence, annealing temperature (T_a) and expected band size (ES) observed in *C. sativus*. Number of alleles (NA) detected in 31 *C. sativus* and 39 *C. melo* accessions, respectively.

Locus name	Location	Repeat motifs	Primer sequences (5'- 3') sense antisense	T_a (°C)	ES (bp)	NA in *C. sativus*	NA in *C. melo*
Ccu1	*trn*L/*trn*F intergenic	$(A)_{11}$	AAAATGGGCAATCCTGAG ATTCCTTTACTAACGCAACG	55	117	2	1
Ccu3	*trn*V/*trn*M intergenic	$(T)_{11}$	CTCTATCGTCCTGCTTCTGC GCCTGACAAAAAGTTCGGT	57	225	2	1
Ccu4	*acc*D/*psa*I intergenic	$(T)_{11}$	TCCACGCTTTCGTTCCCT GCAAGACTCTATGCCGCTAT	57	193	2	3
Ccu5	*clp*P intron	$(T)_{16}$	TAGGATGGTTCCGTTGCT TGTTTTCACGACAGAAGACTC	59	140	2	2
Ccu6	*rpl*14/*rpl*16	$(A)_{11}$-$(A)_{15}$	AAAAGGGTTTGAGGTTGAA TCGGGATAGAAATGTAGAAT	55	175	2	3
Ccu7	*rps*3/*rpl*9 intergenic	$(T)_{14}$	GAACAGCAATAGTATGCCCAA TCTTCGTCGCCGTAGTAAAT	57	233	2	2
Ccu8	*ndh*A intron	$(A)_{15}$	ATCAATAACCCATCGTTTTCA GCATTTCTTTGCTATCTCCC	55	115	2	3
Ccu9	*ycf*1 exon	$(A)_{11}$	TGTCCGTATCCGAAGTCTATC AAAGGCTCTATGCGTGCTC	57	162	1	1
Ccu11	*trn*V/*rps*12 intergenic	$(A)_{13}$	CAGGATCAAACCTATGGGACT GGAATCTGGGCTCTTCTACG	59	112	2	3
Ccu12	*rrn*5/*trn*R intergenic	$(T)_{10}$	CACATCTTCTTAACCCGAAA CCACGTGCTCTAATCCTCT	55	165	1	2
Ccu13	*mat*K exon	$(T)_{10}$	TTGGGAAGATCAAAGAAAG TTTGCGTATATCCAGAAATC	51	230	1	1
Ccu14	*trp*12-*trn*H intergenic	$(A)_{14}$	TAATTTGATTCTTCGTCGC GATGTAGCCAAGTGGATCA	55	228	1	2
Ccu15	*clp*P intron	$(A)_{15}$-$(A)_{12}$	ACTACTCTTTCGATACATAACCT GTCTAGCATTCCCTCACG	51	175	1	2
Ccu16	*rpl*16/*rps*3 intergenic	$(T)_{16}$	CTCTTTCTTTCACCCTTCCAT CATCGCTTCGCATTATCTG	55	172	2	1
Ccu17	*ycf*1 exon	$(T)_{15}$	TGCATTTATCCTTGTTGTACT GAAACACACTACTCATTACCG	55	169	1	2

Table 2. Chloroplast microsatellite loci detected by 15 cpSSR markers among *Cucurbitaceae* species represented by 111 accessions. These species are *Benincasa hispida* (BH), *Citrullus lanatus* (CL), *Cucumis melo* (CM), *Cucumis sativus* (CS), *Cucurbita maxima* (CMA), *Cucurbita moschata* (CMO), *Cucurbita pepo* (CP), *Lagenaria siceraria* (LS), *Lagenaria siceraria* var. *depressa* (LSD), *Luffa cylindrica* (LC), *Momordica charantia* (MC), *Trichosanthes kirilowii* (TK) and *Sechium edule* (SE). The numbers of accessions are in brackets.

Locus	BH (1)	CL (9)	CM (39)	CS (31)	CMA (3)	CMO (8)	CP (2)	LS (6)	LSD (1)	LC (2)	MC (2)	TK (6)	SE (1)
Ccu1	199	117,118	117	115,117	119	117,119	117	117	117	118	117	117,118	118
Ccu3	225	225	225	225,236	229	225	225	225	225	228	228	225	227
Ccu4	340	194,342	187,191,193	187,193	321	328,340	341	340	342	340	340	341	342
Ccu5	139	137,139	140,144	136,140	146,149	139,145	145,148	139,144	139	138	147	139,146	149
Ccu6	180	180,182	172,175,180	172,175	-	173	185	175	175	175	182	163,185	190
Ccu7	233	233,242	233,244	233,234	244	241	233	233	233	233	232	241	233
Ccu8	107	108	107,109,110	107,109	-	110	110	110	108	108	108	110	110
Ccu9	162	162	162	162	162	162	162	162	162	162	162	162	162
Ccu11	110	110	107,108,110	104,106	110	111	108	107	107	111	111	110	110
Ccu12	165	165	163,165	165	165	163,165	165	165	165	165	165	165	165
Ccu13	230	230	230	230	230	230	230	230	230	230	230	230	230
Ccu14	228	228	228,229	226	233	226,231	230	228,236	228	229,238	229,238	230	230
Ccu15	175	175,177	168,175	175	177	172,175	176	175,176	173	175	175	175	175
Ccu16	163	163	163	172,174	164	171,173	163	163	156	166	166	171	163
Ccu17	169	169	169,170	169	169	170	169	169	169	169	169	171	169

manual of silver sequence DNA staining reagents (*Promega*, Madison, WI). The size of the amplified fragments was determined with the software *QUANTITY ONE* (*version 3.0*, *Bio-Rad*) by comparison to the marker ladder.

Screening of *C. sativus* chloroplast genome resulted in identification of 52 SSRs which contained poly(A/T)$_n$ with between 10 and 17 repeats. Because quite short distance (less than 20 bp) was found among some cpSSRs and each of them was not suitable for primer location, only forty one primer pairs were constructed and submitted to synthesis. Of them, 15 primer pairs were selected for absence of non-specific amplification (Table 1). Of the markers, 9 (60 %) and 10 (66.7 %) successfully detected variation in 31 *C. sativus* and 39 *C. melo* accessions, respectively. Each of the polymorphic markers amplified fragments around the expected size, suggesting that the polymorphism mainly arose from number variation of the cpSSR motifs. Two coding region located makers, *i.e.* Ccu9 and Ccu13, displayed intraspecific monomorphism within *C. sativus* or *C. melo*. The number of variants was one or two in *C. sativus* and one to three in *C. melo*. A total of 34 polymorphic loci (an average of 2.3 loci per marker) were identified by the 15 cpSSR markers in *Cucumis*. This value was similar to that found in *Hordeum* (Provan *et al*. 1999) and *Oryza* (Ishii and McCouch 2000) but much lower than in *Lolium* where mean 4.7 alleles per cpSSR were found (McGrath *et al*. 2006). This difference might be resulted from the wide application of wild species during screening of cpSSR variation in the latter case; in present research, 68 out of 70 *Cucumis* accessions were cultivated germplasms, most of which could have common ancestors or chloroplast donors.

In a separate investigation, the allelic diversity of the same set of cpSSR markers was calculated respectively for *C. sativus* and *C. melo* according to the gene diversity value described by Nei (1987). The low diversity values of 0 to 0.437 in *C. sativus* and 0 to 0.528 in *C. melo* were observed (data not shown). The low level of chloroplast polymorphism observed here might reflect the high conservation of the chloroplast-genome sequence of the two species.

Transferability of the cpSSR primers was tested using an additional set of 41 accessions belonging to the family of *Cucurbitaceae*. With the exception of two markers Ccu6 and Ccu8 that had no PCR product in *Cucurbita maxima*, the remaining 13 markers successfully amplified across all accessions tested in present studies (Table 2). At the two loci, *C. maxima* showed null genes (defined as no amplification) which could result from base substitution mutation. Weising and Gardner (1999) sequenced cpSSR-containing regions of some dicotyledonous angiosperms and found that both variable copy numbers of mononucleotide repeats and mutational hotspots in non-coding chloroplast DNA regions were main factors underlying cpSSR variation. Most cpSSR markers produced fragments around expected sizes but the marker Ccu4 was an exception due to generation of much larger size of fragments (> 340 bp) from the tested species excluding *Cucumis*. At this locus, larger insertion/deletion could happen and mask the variations arising from the microsatellite loci. The similar finding has been reported by Bryan *et al*. (1999) in *Solanaceae*. Two cpSSR loci, Ccu9 and Ccu13, which were located in exon region, were monomorphic in all *Cucurbitaceae* plants, but the others (derived from intergenic regions or introns) revealed interspecific variation. This finding implied useful information for primer development that the cpSSR markers derived from non-coding region could have more practicable potential than those targeted coding region in genetic analysis of chloroplast genome.

Intraspecific polymorphisms were also observed within other species besides *C. sativus* and *C. melo*, *i.e. Citrullus lanatus*, *Cucurbita moschata*, *Lagenaria siceraria* and *Trichosanthes kirilowii*. Each of them, which included six to nine individuals, revealed no less than two fragment types at one cpSSR locus or more.

To the best of our knowledge, this is the first report on development of chloroplast microsatellites for *Cucumis* and analysis of their transferability on other *Cucurbitaceae* species. At present, application of these novel cpSSR markers in studying molecular phylogeny of *Cucumis* species is under way in our laboratory.

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