Applications in Plant Sciences 2014 2(3): 1300075

Primer Note

Development of 12 chloroplast microsatellite markers in *Vigna unguiculata* (Fabaceae) and amplification in *Phaseolus vulgaris*[1]

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- *Premise of the study: Vigna unguiculata* is an economically important legume, and the complexity of its variability and evolution needs to be further understood. Based on publicly available databases, we developed chloroplast microsatellite primers to investigate genetic diversity within *V. unguiculata* and its related species *Phaseolus vulgaris*.
- *Methods and Results:* Twelve polymorphic chloroplast microsatellite markers were developed and characterized in 62 *V. unguiculata* individuals. The number of alleles per locus varied between two and four, the unbiased haploid diversity per locus ranged from 0.123 to 0.497, and the polymorphism information content varied from 0.114 to 0.369. In cross-species amplifications, nine of these markers showed polymorphism in 29 *P. vulgaris* individuals.
- *Conclusions:* The newly developed chloroplast microsatellite markers exhibit variation in *V. unguiculata* as well as their transferability in *P. vulgaris*. These markers can be used to investigate genetic diversity and evolution in *V. unguiculata* and *P. vulgaris*.

Key words: chloroplast microsatellite; cross-amplification; Fabaceae; *Phaseolus vulgaris*; *Vigna unguiculata*.

Cowpea (*Vigna unguiculata* (L.) Walp.) ($2n = 2x = 22$), a legume crop of economic importance, is widely distributed in the arid and semiarid regions of Africa, Asia, Europe, Latin America, and some parts of the United States (Citadin et al., 2011). As a member of the legume family, it belongs to Phaseoleae, the same tribe as common bean (*Phaseolus vulgaris* L.). Compared to its close relatives and many other crop species, *V. unguiculata* shows a greater tolerance to drought and has the ability to fix nitrogen in poor soils (Muchero et al., 2009). Its grains are a major source of dietary protein for humans, and cowpea hay is fed to livestock as a nutritious fodder (Badiane et al., 2012). However, even though restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), and simple sequence repeat (SSR) molecular makers have been developed for the cowpea nuclear genome, knowledge of variability and evolution in the chloroplast genome of *V. unguiculata* is limited at the molecular level (Provan et al., 2001; Xu et al., 2010).

Chloroplast microsatellite, or chloroplast simple sequence repeat (cpSSR), markers can be used to detect DNA variability in the chloroplast genome. They have the same characteristics as nuclear microsatellites, including a multiallelic and codominant nature. Moreover, cpSSR markers are found to be polymorphic and transferable among related species because the flanking regions of cpSSR loci are conserved. Of particular importance, cpSSR markers are maternally inherited in most angiosperms, which allow monitoring of influence on population structure by seed-mediated gene flow and pollen flow (Provan et al., 2001). Therefore, they are useful for analysis of population genetics, genetic diversity, paternity analysis, and germplasm resource identification (Provan et al., 2001). In this study, we developed 12 cpSSR markers for *V. unguiculata* and evaluated their transferability to a related legume species, *P. vulgaris*. These results will be helpful for the future exploration and germplasm conservation in both *V. unguiculata* and *P. vulgaris*, although chloroplast microsatellite diversity in *P. vulgaris* has been investigated (Angioi et al., 2009; Desiderio et al., 2013).

METHODS AND RESULTS

The complete chloroplast genome sequence of *V. unguiculata* was downloaded from GenBank (GenBank accession no. NC_018051). The cpSSR loci distributed throughout the *V. unguiculata* chloroplast genome were screened using SSRHunter 1.3 software (Li and Wan, 2005). SSRs were selected based on the length of the core repeat motif (≥10 nucleotides), for example, five units of dinucleotide repeat motifs, four units of trinucleotide repeat motifs, or three units of tetranucleotide repeat motifs. Primer pairs were designed based on the flanking regions of each SSR locus using Primer3 (Rozen and Skaletsky, 2000). The parameters of each primer were set using the following criteria: (1) primer size of 20–24 nucleotides in length; (2) GC content of 40–60%; (3) annealing temperature between 50–60°C; and (4) expected amplicon size of 100–300 bp. In total, 15 cpSSR primer pairs of *V. unguiculata* were designed and synthesized (Sangon, Shanghai, China). Twelve of them showed polymorphic bands

[1]Manuscript received 20 September 2013; revision accepted 5 December 2013.

The authors thank Professor Zeng Chen (George Washington University, Washington, D.C., USA) for his suggestions and improvement to the English. This work was supported by the Wuhan Planning Project of Science and Technology (no. 2013021001010478, no. 201250499145-11) and by the Research Foundation for Talented Scholars of Jianghan University (no. 2012027), Hubei Province, People's Republic of China.

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doi:10.3732/apps.1300075

TABLE 1. Characteristics of 12 polymorphic cpSSR markers developed in *Vigna unguiculata*.

Locus	Repeat motif	Primer sequences (5′–3′)	T_a (°C)	Position[a]	Region	GenBank accession no.	Size range in *V. unguiculata* (bp)	Size range in *P. vulgaris* (bp)
VgcpSSR1	$(TA)_5$	F: GGTGGATGTTTATACCCAATCG R: TCTTTCTGCGATACAAACAAGAA	60	*trnK-rbcL* IGS	LSC	KF662476	190–220	190–196
VgcpSSR2	$(AAT)_5$	F: TTTTCTATGTATGGCGCAACC R: CGGGGATAAAGCTGCCTATT	60	*rbcL-atpB* IGS	LSC	KF662477	180–190	186–190
VgcpSSR3	$(TA)_{12}$	F: AAACCACTCGAATATTATGGAAA R: CCAGTTCAAATCTGGTTCCTG	57	*ndhJ-trnF* IGS	LSC	KF662478	185–305	265–355
VgcpSSR4	$(AT)_5$	F: GAAAAGAACAAGCAAATCCACA R: TGATCCTTACGATGCTTCCTTT	60	*ycf3* exon	LSC	KF662479	180–280	180–280
VgcpSSR5	$(TA)_5$	F: AGCCCACTTTTCCGTAGGTT R: CTTTTCCTTGCCATAATGGTT	58	*psaB-rps14* IGS	LSC	KF662480	190–202	190–202
VgcpSSR7	$(TA)_6$	F: TCAACCATTTCCCAACACCT R: CATCGAGTTCATGGATTTGC	59	*psbD-trnT* IGS	LSC	KF662481	136–196	196
VgcpSSR9	$(TA)_5$	F: TGAAATTTGAAAAACGGGGTA R: AAGCGATACGGATAGATTCCT	57	*trnR-trnS* IGS	LSC	KF662482	144–156	160
VgcpSSR10	$(AT)_5$	F: GGGCTCATTGGCTGTAGAAA R: CCATCTCTCCCCAATTGAAA	59	*trnR-trnS* IGS	LSC	KF662483	150–182	182–186
VgcpSSR11	$(AT)_6$	F: TTTGAGAAGGTTCAATTGTTCG R: TCGGACTCTAGGAAAGGACAA	59	*petA-psbJ* IGS	LSC	KF662484	168–186	168–170
VgcpSSR12	$(AT)_6$	F: GGCCATTTATCCCACTTTCC R: CCAGTCTCTACTGGGGGTTA	56	*psbJ-psbL-psbF* IGS	LSC	KF662485	162–220	170–220
VgcpSSR13	$(TA)_5$	F: TATTGGTTTTGCACCAATCG R: ACCAGGGTGTATGTGCGACT	60	*rpl20-rps12* IGS	LSC	KF662486	162–210	210
VgcpSSR14	$(AT)_5$	F: TGGATCATAATCCTTGAACATCA R: TGCGAAAACAAAGATAAGAAATCA	59	*psaC-ndhE* IGS	SSC	KF662487	162–210	178–180

Note: IGS = intergenic spacer; LSC = long single-copy region; SSC = short single-copy region; T_a = annealing temperature.
[a] Position of each SSR in chloroplast complete genome of *Vigna unguiculata* (GenBank accession number: NC_018051).

in *V. unguiculata* accessions, two were monomorphic, and one primer pair gave no products. The 12 polymorphic markers were used in the following analysis.

A total of 91 samples were used in this study, including 62 *V. unguiculata* accessions and 29 *P. vulgaris* accessions (Appendix 1). All the samples were collected from an agricultural field in Anshan (30.46°N, 113.94°E), Caidian District, Wuhan City, and preserved in Hubei Province Engineering Research Center of Legume Plants, Wuhan, China. Tender young leaves of each sample were collected and stored at −80°C until use. Total DNA was extracted from all the samples using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). The yield and purity of the DNA were measured using a spectrophotometer SP-1910UVPC (Shanghai, China) at an A260/A280-nm wavelength.

Characteristics of cpSSR markers were examined in both *V. unguiculata* and *P. vulgaris*. The same PCR conditions were applied in the two species. The PCR amplifications were performed in a 20-μL reaction mixture containing 1× *Taq* buffer, 30 ng of genomic DNA, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM for each primer, and 0.5 U *Taq* polymerase (MBI Fermentas, Vilnius, Lithuania). The PCR conditions were as follows: an initial denaturation at 94°C for 5 min; followed by 35 cycles of 30 s at 94°C, 30 s at the locus-specific annealing temperature (Table 1), and 40 s at 72°C; and a final extension at 72°C for 5 min. The PCR products were separated using 6% denaturing polyacrylamide gels (Acr : Bis = 19 : 1) and visualized with silver staining. Due to the nonrecombining nature of the chloroplast genome, each pair of chloroplast microsatellite primers was considered as a "locus" at a cpSSR site. Length variants of chloroplast microsatellites at each cpSSR site were treated as alleles. Alleles detected from polymorphic primer pairs were used to generate a chloroplast haplotype of each individual; multilocus haplotypes were obtained by combining alleles from all polymorphic loci. Based on the polymorphic cpSSR markers, the fragment size amplified from each locus was scored by referring to a 20-bp DNA ladder (TaKaRa Biotechnology Co., Dalian, China). The number of alleles (*A*) and unbiased haploid diversity index (*h*) per polymorphic locus were calculated using the software GenAlEx version 6.41 (Peakall and Smouse, 2006). To estimate the informativeness of each SSR marker, the polymorphism information content (PIC) was calculated using the formula described by Botstein et al. (1980).

As shown in Table 2, the characteristics of the 12 polymorphic cpSSR loci are tested in 62 *V. unguiculata* samples. *A* ranged from two to four in *V. unguiculata* (average: 2.75), *h* ranged from 0.123 (VgcpSSR4) to 0.497 (VgcpSSR5) (average: 0.240), and PIC ranged from 0.114 (VgcpSSR4) to 0.369 (VgcpSSR5) (average: 0.211).

The transferability of the 12 *V. unguiculata* cpSSR markers was assessed in a related species, *P. vulgaris*; parameters of genetic variation were evaluated in 29 *P. vulgaris* individuals (the *P. vulgaris* group) (Table 2). All of the 12 cpSSR markers were successfully amplified in the *P. vulgaris* group, and nine showed polymorphisms, with the exception of VgcpSSR7, VgcpSSR9, and VgcpSSR13, which were monomorphic markers. Therefore, it indicated that 75% of these markers can amplify polymorphic bands. In *P. vulgaris*, *A* ranged from one to two, with an average value of 1.75. For each cpSSR locus, *h* was between 0.000 (VgcpSSR7, VgcpSSR9, and VgcpSSR13) and 0.529 (VgcpSSR10 and VgcpSSR14) (average: 0.312). The PIC value varied between 0.183 (VgSSR3) and 0.374 (VgcpSSR2, VgcpSSR10, and VgcpSSR14) (average: 0.312).

CONCLUSIONS

Twelve polymorphic cpSSR markers were developed in *V. unguiculata* and showed high transferability in *P. vulgaris.* Further

TABLE 2. Characterization of the 12 cpSSR markers in *V. unguiculata* and their cross-species amplification in *P. vulgaris*.

	V. unguiculata group			*P. vulgaris* group		
Locus	*A*	*h*	PIC	*A*	*h*	PIC
VgcpSSR1	3	0.210	0.196	2	0.323	0.262
VgcpSSR2	3	0.362	0.303	2	0.516	0.374
VgcpSSR3	2	0.153	0.139	2	0.212	0.183
VgcpSSR4	2	0.123	0.114	2	0.380	0.298
VgcpSSR5	2	0.497	0.369	2	0.467	0.332
VgcpSSR7	2	0.125	0.116	1	0.000	—
VgcpSSR9	2	0.151	0.138	1	0.000	—
VgcpSSR10	4	0.256	0.237	2	0.529	0.374
VgcpSSR11	3	0.202	0.185	2	0.441	0.329
VgcpSSR12	4	0.270	0.255	2	0.349	0.280
VgcpSSR13	3	0.154	0.146	1	0.000	—
VgcpSSR14	3	0.383	0.328	2	0.529	0.374
Average	2.75	0.240	0.211	1.75	0.312	0.312

Note: *A* = number of alleles for each locus; *h* = unbiased haploid diversity; PIC = polymorphism information content.

analyses indicated that the cpSSR markers of *V. unguiculata* could reveal a relatively high level of genetic diversity in both *V. unguiculata* and *P. vulgaris* germplasm. These markers can be used to investigate genetic diversity and evolution in *V. unguiculata* and *P. vulgaris*.

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Appendix 1. Voucher information for legume species used for the cpSSR polymorphism study. All vouchers are deposited at the Hubei Province Engineering Research Center of Legume Plants, Wuhan, China.

Species	Voucher accession no.	Country of origin/source
Phaseolus vulgaris	B48	China
	C-1	China
	C-2	China
	C-3	China
	C-4	China
	C-6	China
	C-7	China
	C-8	China
	C-11	China
	C-12	China
	(13*20)-2	China
	(13*20)-5	China
	(13*20)-10	China
	(13*20)-7	China
	(13*20)-1	China
	(13*20)-9	China
	(13*20)-4	China
	(1*7)-1	China
	(1*7)-2	China
	(1*7)-7	China
	(1*7)-9	China
	(1*7)-10	China
	(1*7)-3	China
	(3*10)-4	China
	(3*10)-5	China
	(3*10)-6	China
	(3*10)-7	China
	(3*10)-8	China
	(3*10)-9	China
Vigna unguiculata	B28	China
	B30	China
	B32	United States
	B34	China
	B35	China
	B36	China
	B37	China
	B39	China
	B42	China
	J2	United States
	J3	United States
	J5	United States
	J7	United States
	J9	Africa
	J11	Mexico
	J13	Germany
	B3	Japan
	B4	China
	B5	China
	B6	China
	B7	China
	B8	China
	B9	China
	B10	China
	B11	China
	B12	China
	B15	China
	B16	China
	B17	China
	B18	China
	B20	China
	A80	China
	A89	China
	A93	China
	A96	China
	A98	China
	A104	China
	A105	China
	A115	China
	A125	China
	A136	China
	A143	China
	A1	China
	A8	China
	A27	China
	A33	China
	A58	China
	A70	China
	A156	China
	A162	China
	A168	China
	A171	China
	A176	China
	A181	China
	A182	China
	A185	China
	A189	China
	A192	China
	A194	China
	A71	China
	A82	China
	A155	China