DEVELOPMENT OF 12 CHLOROPLAST MICROSATellite MARKERS IN VIGNA UNGUICULATA (FABACEAE) AND AMPLIFICATION IN PHASEOLUS VULGARIS

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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). How- ever, 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 poly- morphic 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 (Li and Wan, 2005). 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
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 V. unguiculata accessions (Appendix 1). All the samples were collected from an agricultural field in Anshan (30.46°N, 123.4°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.

Twelve polymorphic cpSSR markers were developed 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 MgCl₂, 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 nonrecombinant 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 haplotype diversity index (h) per polymorphic locus were calculated using the software GenAIEx 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 (VgcpSSR3) 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

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

| Locus      | Repeat motif | Primer sequences (5′-3′) | Tₘ(°C) | Position | Region | GenBank accession no. | Size range in V. unguiculata (bp) | Size range in P. vulgaris (bp) |
|------------|--------------|--------------------------|--------|----------|--------|------------------------|---------------------------------|---------------------------------|
| VgcpSSR1   | (TA)₃        | F: GGTGGAATTTTACCAACATGC | 60     | trnK-rbcL IGS | LSC | KF662476 | 190–220 | 190–196 |
| VgcpSSR2   | (AT)₃        | R: TCTTCTCTGATACAAACCAAGA | 59     | rbcL-atpB IGS | LSC | KF662477 | 180–190 | 186–190 |
| VgcpSSR3   | (TA)₁₂      | F: AAAACACTGTATATATTGAGGA | 57     | ndh-t-trnF IGS | LSC | KF662478 | 185–305 | 265–355 |
| VgcpSSR4   | (AT)₃        | F: GAAAAAAGAACAACAACTCACA | 60     | ycf3 exon | LSC | KF662479 | 180–280 | 180–280 |
| VgcpSSR5   | (TA)₃        | F: AGGCCGCTTTTCGATGTTT | 58     | psbB-rps14 IGS | LSC | KF662480 | 190–202 | 190–202 |
| VgcpSSR7   | (TA)₆        | F: TCAACATTCTTCCAAACACCT | 59     | psbD-trnT IGS | LSC | KF662481 | 136–196 | 196 |
| VgcpSSR9   | (TA)₃        | F: TGAATAATGGAAAGGCTTTA | 57     | trnK-trnS IGS | LSC | KF662482 | 144–156 | 160 |
| VgcpSSR10  | (AT)₃        | F: GGGCTACATGTCAGTAGAAA | 59     | trnR-trnS IGS | LSC | KF662483 | 150–182 | 182–186 |
| VgcpSSR11  | (AT)₆        | F: TGGGAAAGGTCTCAACCTTGCG | 59     | petL-psbJ IGS | LSC | KF662484 | 168–186 | 168–170 |
| VgcpSSR12  | (AT)₂        | F: GCCATTATCTCCTACATCCCC | 56     | psbF-psbL-psbF IGS | LSC | KF662485 | 160–220 | 170–220 |
| VgcpSSR13  | (TA)₃        | F: YTATGGTTTGGCACAATCGT | 60     | rpl20-rps12 IGS | LSC | KF662486 | 160–210 | 210 |
| VgcpSSR14  | (AT)₃        | F: TGGATGATATATCGAGGAT | 59     | psbA-ndhE IGS | SSC | KF662487 | 160–210 | 178–180 |

Note: IGS = intergenic spacer; LSC = long single-copy region; SSC = short single-copy region; Aₘ = annealing temperature.

*Position of each SSR in chloroplast complete genome of Vigna unguiculata (GenBank accession number: NC_018051).*

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

| Locus      | V. unguiculata group | P. vulgaris group |
|------------|----------------------|-------------------|
| A          | h                    | PIC               |
| A          | h                    | PIC               |
| VgcpSSR1   | 3                    | 0.210             | 0.196 | 0.323 | 0.262 |
| VgcpSSR2   | 3.622                | 0.303             | 0.516 | 0.374 |
| VgcpSSR3   | 0.153                | 0.139             | 0.212 | 0.183 |
| VgcpSSR4   | 0.123                | 0.114             | 0.380 | 0.298 |
| VgcpSSR5   | 0.497                | 0.369             | 0.467 | 0.332 |
| VgcpSSR7   | 0.125                | 0.116             | 0.000 | —     |
| VgcpSSR9   | 0.151                | 0.138             | 0.000 | —     |
| VgcpSSR10  | 0.256                | 0.237             | 0.529 | 0.374 |
| VgcpSSR11  | 0.202                | 0.185             | 0.441 | 0.329 |
| VgcpSSR12  | 0.270                | 0.255             | 0.349 | 0.280 |
| VgcpSSR13  | 0.154                | 0.146             | 0.000 | —     |
| VgcpSSR14  | 0.383                | 0.328             | 0.529 | 0.374 |

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

http://www.bionose.com/loi/apps
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                    |
|                          | 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                    |
| *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                    |