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Isolation and characterization of microsatellite loci for the herbaceous tuber crop, *Amorphophallus konjac* (Araceae)

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ABSTRACT. *Amorphophallus konjac* is an herbaceous tuber crop with tremendous potential for commercial development. We report the development of microsatellite primers for this important crop species. Thirteen polymorphic microsatellite markers were developed and tested in two populations of *A. konjac* from the Wuling Mountain Region (WL population) and the Yunnan Province (YN population) in China. The number of alleles per locus ranged from 1 to 7; the observed and expected heterozygosities ranged from 0 to 1 and from 0 to 0.844, respectively, in the two populations. These microsatellite markers will facilitate further studies in population genetics and utilization of *A. konjac*.

Key words: *Amorphophallus konjac*; Microsatellite; Tuber crop; Population genetics

INTRODUCTION

The genus *Amorphophallus* includes more than 150 species and is distributed mainly in India, the Indo-China Peninsula, south China, southeast Asia, and Africa (Gao, 2004). Plants of this genus have long been used in tropical and subtropical Asia as a food source and as a traditional medicine (Chua et al., 2010). A total of 9 *Amorphophallus* species have been used as food, medicine, fodder, and for wine production (Gao, 2004). One of the most widely utilized species is *Amorphophallus konjac* K. Koch ex N.E.Br., which has been used in China, Japan and southeast Asia for thousands of years. *A. konjac* is an herbaceous perennial species that has an underground stem in the form of a tuber. The soluble fiber extracted from the tubers, commonly known as konjac glucomannan, is used as a food additive and in the development of dietary supplements or nutraceuticals (Chua et al., 2010).

In China, *A. konjac* is mainly distributed in the Sichuan, Hubei, Yunnan, Guizhou, Guangdong, Guangxi, and Fujian Provinces. This species has been recognized as one of the most important crops and has tremendous potential for commercial development. Understanding the genetic basis of germplasm resources is essential for the efficient evaluation, conservation and utilization of authentic and superior crop materials. However, only a few studies have been reported regarding the genetic diversity of *A. konjac* to date, and these studies were all based on dominant molecular markers, e.g., random amplification of polymorphic DNA and inter-simple sequence repeat (Zhang et al., 2001; Teng et al., 2006; Xuan, 2010). In addition, these studies were all focused on the phylogenetic relationships of germplasm resources of *Amorphophallus* species including *A. konjac*, and no study has focused specifically on the population genetics of *A. konjac*. Herein, we report the development and characterization of 13 polymorphic microsatellite loci from *A. konjac* to facilitate the ongoing studies of genetic diversity and exploration of this species.

MATERIAL AND METHODS

A dinucleotide-enriched microsatellite genomic library was constructed and screened for *A. konjac* using a fast isolation by amplified fragment length polymorphism of sequences containing repeats (FIASCO) protocol, with some modifications (Zane et al., 2002). Total DNA was isolated from 1 dry leaf tissue sample of *A. konjac* from the Wuling Mountain Region [WL population, 108°58'30" E/29°28'36" N; Voucher specimen was deposited in the herbarium of Wuhan University, Wuhan, China (WU), and the accession number was C. Pan et al. W1108] of Central China using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). Approximately 250 ng genomic DNA was digested with *Mse*I (New England Biolabs, Beverly, MA, USA), and then ligated to an *Mse*I adaptor pair (5'-TACTCAGGACTCAT-3'/5'-GACGATGAGTCCTGAG-3') using T4 DNA ligase (Fermentas, Vilnius, Lithuania) and diluted (1:5). Then, 5 µL diluted digestion-ligation mixture was directly amplified with 1 µL 25 µM *Mse*I-N primer (5'-GATGAGTCCTGAGTAAN-3') in a 20-µL volume containing 1 U *Taq* polymerase (Takara, Shiga, Japan), 2 µL 10X PCR buffer, 0.4 µL 2.5 mM of each dNTPs, following the PCR program: 95°C for 3 min, 20 cycles of 94°C for 30 s, 53°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 5 min.

Approximately 500-1000 ng amplified DNA was hybridized with 200 pM 5'-biotinylated $(AC)_{15}$ oligonucleotide in a total volume of 250 µL 4.2X SSC (saline sodium citrate) and

0.07% SDS (sodium dodecyl sulfate). The mixture was incubated at 95°C for 5 min, followed by annealing at 68°C for 2 h and cooling to room temperature. The hybridization mixture was enriched using 600 µL streptavidin-coated beads (Promega, Madison, WI, USA), 3 nonstringent and 3 stringent washes were carried out following the protocol of Zane et al. (2002). DNA containing repeats was amplified for 25 cycles with *Mse*I-N primers. The PCR conditions (including the reagents used and the thermal cycler program) were the same as those described above. The PCR product was purified with E.Z.N.A.® Gel Extraction Kit (Omega Bio-tek, Norcross, GA, USA) and ligated into a pMD18-T plasmid vector (Takara) and transformed into DH5α competent cells (Takara). Transformants were plated and insert-containing clones were selected by blue/white screening.

Ninety-one positive clones (plasmid inserts) were sequenced with M13R or M13F primers using the ABI Prism BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 3730 automated DNA sequencer (Applied Biosystems) following the manufacturer protocol. A total of 63 sequences possessing microsatellite motifs were obtained and then subjected to primer design using PRIMER 3 version 0.4.0 (Rozen and Skaletsky, 2000).

To test the polymorphism of the microsatellite primers, 225 germplasm resources of *A. konjac* from the WL population (N = 190; 108°58'35" E/29°28'30" N) in Central China and the YN population (N = 35; 101°21'12" E/21°52'50" N) in Yunnan Province of southwest China were genotyped. Voucher specimens were deposited in the herbarium of WU, and the accession numbers were C. Pan et al. W1101 and C. Pan et al. Y1108. PCR amplifications were performed in 20-µL reaction mixtures containing 1 U *Taq* polymerase (Takara), 2 µL 10X PCR buffer, 0.3 µL 2.5 mM of each dNTPs, 1 µL 10 µM of each primer and approximately 10 ng genomic DNA. Cycling conditions were 5 min at 94°C, followed by 30 cycles of 50 s at 94°C, 50 s annealing at 50°-60°C, a 1-min extension at 72°C, and a final extension step of 7 min at 72°C. The annealing temperatures were locus-specific (see Table 1). Products were resolved on a 6% polyacrylamide denaturing gel and visualized by silver staining. Product sizes were determined by comparison to a 25-bp DNA ladder (Promega). The number of observed alleles per locus and the observed (H_O) and expected (H_E) heterozygosities for each locus for the two test populations of *A. konjac* were calculated using the Arlequin ver. 3.1 program (Excoffier et al., 2005). The calculations were only conducted on the unique genets.

RESULTS AND DISCUSSION

A total of 13 of the 63 primer pairs successfully amplified clear and consistent DNA fragments across all 225 samples (Table 1). Considering that *A. konjac* is a diploid species (Gao, 2004), we discarded the remaining primer pairs since some failed to amplify and others produced multi-banding patterns. All of the 13 primer pairs were polymorphic and the characteristics of these loci are presented in Table 1. Only 30 genets among 190 individuals from the WL population and 31 genets among 35 individuals from the YN population were revealed when we used all 13 loci. Based on the calculations performed on the genets, the number of alleles per locus ranged from 1 to 7. The H_O and H_E ranged from 0 to 1 and from 0 to 0.844, respectively (Table 2). This set of novel markers represents a set of highly polymorphic, selectively neutral and co-dominant markers, which are potentially useful for further studies of population genetics, exploration and utilization of this important crop.

Table 1. Characterization of the 13 microsatellite primers developed in *Amorphophallus konjac* (*Amor*).

Locus	Primer sequence (5'-3')	Repeat motif	Size	Ta (°C)	GenBank accession No.
Amor 22	F: AATTAGAGGTGAATAGGGAGG R: AAGTATTGATAAAAGTTTGCCA	$(AG)_5(GA)_5\ldots(GA)_5\ (AG)_7\ldots(AG)_5$	169	60	JF710594
Amor 33	F: TTAAGTATTGAACATCTGATGTACTC R: AATGCTCCACTATGACGTGA	$(TG)_5(GT)_9(TG)_5$	176	58	JF710595
Amor 34	F: GTGGGGAGTGGAGGTGGTTG R: GAGATTCCATTAGTTGGGTGAGAAA	$(GA)_{19}$	154	60	JF710596
Amor 36	F: CCACCCCTCACCGACAG R: TCCTTCAATTTCACATCCTTCT	$(CA)_6\ldots(CA)_8\ldots(CA)_6$	197	54	JF710597
Amor 41	F: GGTTGCACAGGCTCAGATTGT R: GACGGGCTCCCTATGTCCAC	$(GT)_{11}\ldots(GT)_6\ (TG)_6$	251	58	JF710598
Amor 43	F: GGGAGTCTTGGGAGGTGAAA R: AAATTTGAGCCATTGAGTGCA	$(AG)_{10}(GA)_{11}(AG)_5$	187	54	JF710599
Amor 46	F: CGTCCACCCCTCACTAAACCCG R: CGCCTGCGTGCGGTCGTAT	$(CA)_7(AC)_5\ldots(AC)_9\ldots(AC)_5$	266	60	JF710600
Amor 51	F: TTAATGAGGTGGTTGGGAGGT R: AAGCAAAATTTGAGCCATTGA	$(GA)_7(AG)_6$	153	60	JF710601
Amor 53	F: TAAGGTGATTCGGTTGCAAAT R: TTTGTGTGCATGTGTTGTGAA	$(CA)_8\ldots(CA)_6$	202	54	JF710602
Amor 54	F: CAGATCCAGATGCAACCCTAA R: TCCGCTCAGTATCTGACCATT	$(TG)_7\ldots(TG)_7$	170	60	JF710603
Amor 55	F: TAGGACCTAGGCATCGAGGAT R: TTAAGGACGTGGCAGTGAGA	$(TG)_{14}$	150	54	JF710604
Amor 57	F: GATGGAGTTGTGACTCCGTGT R: GAGGCCTTGCCTCAAACTACT	$(GT)_{14}$	107	50	JF710605
Amor 58	F: AAGAAGGGTGATTAGGGAGGA R: TTAAACCCTCAAGTATTCTCTCTC	$(AG)_6(GA)_8$	120	50	JF710606

F = forward primer; R = reverse primer; Ta = annealing temperature.

Table 2. Results of primer screening in two populations of *Amorphophallus konjac* (*Amor*).

Locus	*A. konjac*					
	WL (N = 30)			YN (N = 31)		
	N_A	H_O	H_E	N_A	H_O	H_E
Amor 22	4	0.93	0.586	4	0.52	0.615
Amor 33	2	0	0.075	1	0	0
Amor 34	7	0.867	0.808	6	0.807	0.633
Amor 36	4	1	0.629	4	1	0.802
Amor 41	3	0	0.492	3	0	0.659
Amor 43	3	0.042	0.196	2	0.067	0.287
Amor 46	4	1	0.546	4	1	0.577
Amor 51	4	0.95	0.745	2	1	0.6
Amor 53	2	1	0.517	2	1	0.6
Amor 54	3	0.85	0.535	5	1	0.844
Amor 55	3	0.182	0.173	2	0.154	0.271
Amor 57	2	0.895	0.50	2	0.5	0.391
Amor 58	3	0.091	0.172	2	0.083	0.083

WL = Wuling Mountain population; YN = Yunnan Province population; N_A = number of observed alleles per locus; H_O = observed heterozygosity; H_E = expected heterozygosity.

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