Development of polymorphic microsatellite markers for *Dioscorea zingiberensis* and cross-amplification in other *Dioscorea* species

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ABSTRACT. *Dioscorea zingiberensis* C.H. Wright (Dioscoreaceae) is an endemic species in central and southwestern China. In order to study the genetic diversity and population structure of this species, 19 novel polymorphic microsatellite loci were developed using a dual-suppression PCR technique. The number of alleles per locus ranged from 3 to 21, with an average of 9.53. All the markers showed high transferability in cross-species amplification in other species of sect. *Stenophora*.

Key words: *Dioscorea zingiberensis*; *Dioscorea* sect. *Stenophora*; Microsatellite; Transferability

INTRODUCTION

Dioscorea zingiberensis C.H. Wright (Dioscoreaceae) is an endemic species in central and southwestern China (Ting and Gilbert, 2000). As the main resource of diosgenin, it is considered to be an extremely important traditional medicine (Ting et al., 1985). The wild populations have declined extensively as a result of overexploitation. In order to better understand the genetic diversity and population structure of *D. zingiberensis*, microsatellite loci were isolated using a dual-suppression PCR technique (Lian et al., 2006), and the cross-species amplification was tested in 10 other species of sect. *Stenophora*.

MATERIAL AND METHODS

Two populations of *D. zingiberensis* were collected in Mt. Wudang and Ankang, China. Ten species of sect. *Stenophora* were collected for testing the cross-species amplification. Voucher specimens were deposited in the Herbarium of Institute of Botany, Jiangsu Province and Chinese Academy of Sciences (NAS) (Table 1).

Table 1. Material information.

Species	Location	Voucher
Dioscorea zingiberensis C.H. Wright	Ankang, Shaanxi	0648768
	Mt. Wudang, Hubei	0648769
D. collettii var. *hypoglauca* (Palibin) C. Pei & C.T. Ting	Hengshan, Hu'nan	0648579
	Yandangshan, Zhejiang	200909061
D. nipponica ssp *rosthornii* (Prain & Burkill) C.T. Ting	Liushan, Gansu	0648571
D. nipponica ssp *nipponica* Makino	Menghai, Yunnan	0648570
	Guangfo, Shaanxi	200909011
D. futschauensis Uline ex R. Knuth	Qingyunshan, Fujian	0648580
D. banzhuana C. Pei & C.T. Ting	Mengzi, Yunnan	0648582
D. simulans Prain & Burkill	Guilin, Guangxi	0648583
D. spongiosa J.Q. Xi, M. Mizuno & W.L. Zhao	Hengshan, Hu'nan	0648581
D. deltoidea Wallich ex Grisebach	Deqin, Yunnan	0648575
D. sinoparviflora C.T. Ting, M.G. Gilbert & Turland	Honghe, Yunnan	0648574

Genomic DNA was extracted from fresh leaves of *D. zingiberensis* using a modified hexadecyltrimethylammonium bromide (CTAB) method (Doyle, 1991). One individual from the Ankang population was used to generate microsatellite sequences. The genomic DNA samples were digested with the *Afa*I restriction enzyme (TaKaRa), and restricted fragments were ligated with an adaptor (upper-lian: 5'-GTA ATA CGA CTC ACT ATA GGG CAC GCG TGG TCG ACG GCC CGG GCT GGT-3'; lower-lian: 5'-ACC AGC CC-NH_2-3') by use of T4 ligase (TaKaRa). Fragments were amplified using compound single sequence repeat (SSR) primers $(AC)_6$, $(AG)_5$, or $(TC)_6(AC)_5$ and an adaptor primer, AP_2 (5'-CTA TAG GGC ACG CGT GGT-3'). PCR amplification was performed in a final volume of 50 µL, consisting of 25 to 50 ng DNA, 1X PCR buffer (containing

Mg^{2+}), 0.2 mM dNTPs, 0.5 μM compound SSR primer and AP_2, and 0.5 U Ex *Taq* polymerase (TaKaRa). The PCR amplification conditions were as follows: an initial denaturation at 94°C for 9 min, annealing at 62°C for 30 s, and extension at 72°C for 1 min; 5 cycles of denaturation at 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and 72°C for 1 min; and a final cycle of denaturation at 94°C for 30 s, 60°C for 30 s, and 72°C for 5 min. The amplified fragments were purified using a DNA gel product recycling kit. Then, the purified fragments were ligated into a pMD-19T vector (TaKaRa). The plasmids were transformed into competent *Escherichia coli* DH5α cells (TaKaRa), and the transformed cells were cultivated on agar medium containing 100 μg/μL Ampicillinum natricum. The cloned fragments were amplified from the plasmid DNA of positive clones using the M13-47/ RV-M universal primers (M13-47: 5'-CGC CAG GGT TTT CCC AGT CAC GAC-3'; RV-M: 5'-GAG CGG ATA ACA ATT TCA CAC AGG-3'). Positive clones were obtained and sequenced on an ABI Prism 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were edited by the Sequencher 4.5 (Gene Coding, Ann Arbor, MI, USA). For each fragment containing compound SSR sequences at one end, a specific primer was designed from the sequence flanking the compound SSR, using Primer Premier 5 (Clarke and Gorley, 2001).

To examine the effectiveness and polymorphism of the primers, 26 individuals from Mt. Wudang and 22 individuals from Ankang were collected, respectively. The compound SSR primers were labeled with fluorescence dyes (6-FAM or HEX). The amplified products were analyzed by fluorescence capillary electrophoresis on an ABI Prism 3730 automated DNA sequencer (Applied Biosystems), and the data were compiled and scored using GeneMaker 1.95 (Soft-Genetics, State College, PA, USA). Cervus 2.0 (Kalinowski et al., 2007) was used to calculate the N_A (number of alleles per locus), H_O (observed heterozygosity), and H_E (expected heterozygosity). Genepop (http://genepop.curtin.edu.au/) was employed to test the Hardy-Weinberg equilibrium and linkage disequilibria (LD) between pairs of loci with Bonferroni correction for multiple testing (Rice, 1989). Cross-amplifications were done to test the transferability of the 19 loci in 10 species of sect. *Stenophora*, and the amplified fragments were checked by 1.5% agarose gel electrophoresis.

RESULTS

Nineteen microsatellite markers were isolated from *D. zingiberensis* (Table 2). The N_A per locus ranged from 3 to 21, with an average of 9.53 alleles per locus. The H_O values ranged from 0.125 to 0.708, with an average of 0.328, whereas the H_E values ranged from 0.511 to 0.940, with an average of 0.750. The polymorphism information content of the 19 microsatellite loci ranged from 0.486 to 0.926, with an average of 0.713. None of microsatellite loci deviated significantly from Hardy-Weinberg equilibrium (P < 0.01). The pairwise LD between the 19 pairs of loci was not significance (P < 0.001). Cross-amplifications results showed that the novel microsatellite loci developed had high transferability in sect. *Stenophora* (Table 3).

Table 2. Characteristics of 19 microsatellite loci developed for *Dioscorea zingiberensis*.

Locus	Primer sequence (5'-3')	Repeat motif	Ta (°C)	Size (bp)	N_A	H_O	H_E	PIC	GenBank accession No.
DzSSR1	F: $(TC)_6(AC)_5$ R: ATTTTTAATCTTTTTTTCCTCC	$(TC)_6(AC)_{12}A(AAAC)_7$	45.2	96	7	0.333	0.511	0.486	JX235677
DzSSR2	F: $(AC)_6(AG)_5$ R: TAGGAATCGGCATTTGAGAGCT	$(AC)_6(AG)_{19}$	48.6	160	9	0.292	0.826	0.795	JX235678
DzSSR3	F: $(AC)_6(AG)_5$ R: TCAAAAATACTAACAAAACCCT	$(AC)_6(AG)_{10}$	46	78	15	0.438	0.907	0.890	JX235679
DzSSR4	F: $(AC)_6(AG)_5$ R: TCAGAAGGTCATCGTCATCA	$(AC)_6(AG)_{10}$	50	152	7	0.208	0.719	0.664	JX235680
DzSSR5	F: $(AC)_6(AG)_5$ R: AAAGACTTCATTCCAGAGACAC	$(AC)_6(AG)_{22}$	49.1	160	21	0.708	0.940	0.926	JX235681
DzSSR6	F: $(AC)_6(AG)_5$ R: AACAGAAGGGCAAGAACC	$(AC)_6(AG)_5$	50.9	136	7	0.271	0.708	0.654	JX235682
DzSSR7	F: $(AC)_6(AG)_5$ R: TTCCACCGACAACCCGAC	$(AC)_6(AG)_5$	49.8	154	8	0.375	0.769	0.723	JX235683
DzSSR8	F: $(AC)_6(AG)_5$ R: ATGAAACAAGAAAGAAACAAAG	$(AC)_6(AG)_{20}$	54	296	8	0.417	0.761	0.729	JX235684
DzSSR9	F: $(AC)_6(AG)_5$ R: CAAAAGACAGAGAAGCACATAC	$(AC)_6(AG)_6$	50.1	188	12	0.313	0.856	0.831	JX235685
DzSSR10	F: $(AC)_6(AG)_5$ R: TCCAGATAATAGGTAGGAACATA	$(AC)_6(AG)_5$	47.2	163	8	0.458	0.759	0.721	JX235686
DzSSR11	F: $(AC)_6(AG)_5$ R: GGCTGGTACTTTGCTTGC	$(AC)_6(AG)_6$	48.2	150	6	0.146	0.519	0.487	JX235687
DzSSR12	F: $(AC)_6(AG)_5$ R: TGGATGCTTGAGATGAGG	$(AC)_6(AG)_{15}$	49.1	99	11	0.313	0.768	0.739	JX235688
DzSSR13	F: $(AC)_6(AG)_5$ R: TTCACCACCAACAGCATT	$(AC)_6(AG)_{13}$	47.7	107	3	0.333	0.575	0.477	JX235689
DzSSR14	F: $(AC)_6(AG)_5$ R: ATCTTCAGGGACCTCAACT	$(AC)_6(AG)_{11}$	46.8	87	8	0.271	0.785	0.747	JX235690
DzSSR15	F: $(AC)_6(AG)_5$ R: CTTTGCTTGCTTCTTCATC	$(AC)_6(AG)_5$	47.5	140	7	0.125	0.589	0.543	JX235691
DzSSR16	F: $(AC)_6(AG)_5$ R: CATAGGTTTATTCTTGGTGC	$(AC)_6(AG)_{14}$	50.3	131	10	0.146	0.772	0.740	JX235692
DzSSR17	F: $(AC)_6(AG)_5$ R: AAAATACTAACAAAACCCTACAC	$(AC)_6(AG)_{19}$	47.1	93	18	0.146	0.906	0.889	JX235693
DzSSR18	F: $(AC)_6(AG)_5$ R: TAGCCCAGTATGGAACAC	$(AC)_6(AG)_{14}$	46.8	192	8	0.438	0.786	0.754	JX235694
DzSSR19	F: $(AC)_6(AG)_5$ R: CCAAACCATACCCACAGC	$(AC)_6(AG)_{16}$	51	97	8	0.500	0.793	0.759	JX235695
Mean					9.53	0.328	0.750	0.713	

Ta = optional annealing temperature; N_A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; PIC = polymorphic information content.

Table 3. Results of cross-species amplification in 10 species in *Dioscorea* sect. *Stenophora*.

Species	Voucher	SSR primer																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
D. collettii var. *hypoglauca*	0648579	+	-	+	+	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-
D. collettii var. *hypoglauca*	200909061	+	-	+	+	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-
D. nipponica ssp *rosthornii*	0648571	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-
D. nipponica ssp *nipponica*	0648570	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
D. nipponica ssp *nipponica*	200909011	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D. futschauensis	0648580	+	-	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	-	-
D. banzhuana	0648582	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	-	-
D. simulans	0648583	+	-	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+	+
D. spongiosa	0648581	+	-	+	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	-
D. deltoidea	0648575	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D. sinoparviflora	0648574	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

(+) = successful amplification with a size similar to that of *Dioscorea zingiberensis*; (-) = no amplification.

DISCUSSION

The development of microsatellite markers for *D. zingiberensis* is essential for ongoing research on genetic diversity and population structure. The information generated is of great importance for the conservation of the genetic variability of this species, as well as other species in sect. *Stenophora*.

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