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BRIEF COMMUNICATION

Development of thirty new polymorphic microsatellite primers for *Paeonia suffruticosa*

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Abstract

Four new microsatellite primer pairs were developed in tree peony (*Paeonia suffruticosa*) based on the database mining and other twenty-six primer pairs by fast isolation by AFLP of sequences containing repeats (FIASCO) method. The polymorphism of each locus was further evaluated in 40 individuals of one population plus 5 tree peony related species. The number of alleles per locus ranged from 3 to 7 and the expected (H_e) and observed (H_o) heterozygosity at each locus ranged from 0.42 to 0.78 and 0.28 to 0.59, respectively. These microsatellite markers will be useful for investigating genetic diversity and studies of population genetic structure of tree peony.

Additional key words: database mining, FIASCO, SSR, tree peony.

Tree peony (*Paeonia suffruticosa*) is one of the famous horticultural plants in the world. In order to protect the valuable wild species and better understanding and sustainable use of the genetic resources of tree peonies, it is crucial to use suitable molecular markers. Several recent studies have documented genetic diversity and constructed genetic relationships among cultivars using DNA molecular markers, such as amplified fragment length polymorphism (AFLP; Hou *et al*. 2006), inter-simple sequence repeat (ISSR; Suo *et al*. 2005), random amplified polymorphic DNA (RAPD; Hosoki *et al.* 1997) and sequence-related amplified polymorphism (SRAP; Han *et al.* 2008, Guo *et al.* 2009).

Microsatellites, also described as polymorphic simple sequence repeats (SSRs), are widely distributed, short, tandemly repeated sequences of 1 - 6 nucleotides. Microsatellites have become the markers of choice for genetic mapping, genotyping, fingerprinting and genetic diversity studies in plants (Akkak *et al.* 2009, Akritidis *et al.* 2009). At present, a lot of methods were available to develop microsatellite markers, among which a time and cost effective method is fast isolation by AFLP of sequences containing repeats (FIASCO) presented by Zane *et al.* (2002). Where abundant sequence data is already available, it is more efficient to use computation tools to identify SSR loci.

Two methods were employed to develop micro-satellite primers. First, the tree peony sequences data were exploited using the software of SSR primer (Jewell *et al.* 2006) in January 2007. There were 362 sequences altogether and 24 sequences were identified, which contained the SSR motifs. Twenty-four primer pairs were designed and used to amplify in 40 individuals of tree peony from one population in Song County, Henan Province, China and 5 tree peony related species, *i.e*., *P. jishanensis*, *P. qiui*, *P. ostii*, *P. rockii* and *P. ludlowii* which were collected from Luoyang Chinese National Genbank of Tree Peony. Only four primer pairs produced clear, stable and polymorphic bands (PSMP1, PSMP2, PSMP3, PSMP7; Table 1).

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Abbreviations: AFLP - amplified fragment length polymorphism; CTAB - cetyltrimethylammonium bromide; FIASCO - fast isolation by AFLP of sequences containing repeats; ISSR - inter-simple sequence repeat; RAPD - random amplified polymorphic DNA; SRAP - sequence-related amplified polymorphism; SSR - microsatellite or simple sequence repeat.

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Table 1. Primers sequence and characteristics of microsatellite loci in tree peony. Annealing temperature (Ta,), size range of fragments, number of alleles (N_A) in *P. suffruticosa* (*P.s*.) and including relative species (r.s.), observed (H_o) and expected (H_e) heterozygosities are based on a sample of 40 individuals of one tree peony population.

Locus	Repeat motif	Primer sequences (5′–3′)	Ta [℃]	Size [bp]	N_A(*P.s*)	N_A (r.s)	H_o	H_e	GenBank No.
PAC38	$(TG)_8$	F:GATGGGTATGATTGTGAGCA	55	373	5	7	0.46	0.65	GQ480167
		R:GTTCCTGTGGTTTGACTTTC	55						
PAC36	$(AC)_{15}$	F:CTGCAGGTCGACGATTAC	57	389	3	4	0.55	0.71	GQ480168
		R:ATCGTGTATGTGTGATGGGT	55						
PAC28	$(AC)_{17}$	F:CTGCAGGTCGACGATTAC	57	247	5	7	0.32	0.67	GQ480169
		R:AGTCCTGAGTAACATTGCCT	55						
PAC37	$(CA)_{12}$,$(AC)_{11}$	F:GAGATTGATGAGTCCTGAGTAA	56	252	3	5	0.43	0.50	GQ480170
		R:TGAATACCCAGTGGAGTTGA	55						
PC1	$(AG)_7$	F:CTACCCACGACCCTTTTGAG	59	243	5	5	0.56	0.71	GQ480171
		R:AGCACTCTCACAACTTTCATAC	56						
PC2	$(TC)_{16}$	F:AAATCACAACACTCCTCACC	55	318	3	4	0.43	0.55	GQ480172
		R:CTTCTCCAGCGTAATCCATA	55						
PC15	$(GA)_9$	F:TAGAGATTGATGAGTCCTGAGT	56	357	6	6	0.40	0.58	GQ480173
		R:AACTCCAGATGATGTTTGAATA	52						
PC4	$(AC)_6$	F:GAGATTGATGAGTCCTGAGTAA	52	276	4	4	0.38	0.59	GQ480174
		R:TGAGAAAAGTGGGAGTGTTG	56						
PC6	$(TC)_{19}$	F:TCTTTCCATTTTCATAGATTTT	48	313	4	5	0.45	0.59	GQ480175
		R:CAAATAAACCAACACCATAAGA	52						
PC7	$(GA)_8$	F:TTTTTCTGGAGGCTACGG	55	241	4	6	0.58	0.73	GQ480176
		R:TATCCAGATTTATCCTCTCACC	56						
PC8	$(CT)_6$	F:GATTGATGAGTCCTGAGTAACC	55	285	5	7	0.49	0.72	GQ480177
		R:GAAGAAAACGGAGAAAAGGT	57						
PC9	$(AG)_{16}$	F:GAAATACTCGGGACGCAG	57	317	4	6	0.35	0.57	GQ480178
		R:TTCTCCCAAGCAAAAGGT	52						
PC11	$(CT)_{14}$,$(TC)_{16}$	F:GAGTCCTGAGTAACCCAACA	57	382	5	6	0.55	0.75	GQ480179
		R:CAAACACCAAGACCGAAT	52						
PC13	$(AG)_{13}$,$(GA)_{10}$	F:AGCAAAAAGGGAGAAGTAAG	53	369	5	5	0.49	0.67	GQ480180
		R:ATTATGGCGAGTTATTTGGA	51						
PAC51	$(AT)_5$，$(CA)_{16}$	F:AGAGATTGATGAGTCCTGAGTA	56	298	5	6	0.57	0.73	GQ480181
		R:TGAAGGTTTGTAAAGTAGGAGA	54						
PAC52	$(TA)_5$	F:CCAAACCCAAACAGAACC	54	327	6	6	0.58	0.62	GQ480182
		R:CCGATACACCCATCCTCA	56						
PAC54	$(GT)_{19}$,$(TG)_{14}$	F:GATACTTAGTTCCAACCTGTGA	56	375	3	5	0.32	0.60	GQ480183
		R:TGGCGATAAACTGAGTGAAA	53						
PAC55	$(AC)_{16}$,$(AC)_{14}$	F:ACTACCCAGGCGATGTGC	59	257	3	3	0.28	0.47	GQ480184
		R:AAGGTGGTGGAGGAAGAT	55						
PAC58	$(AC)_5$	F:TAGAGATTTGATGAGTCCTGAG	57	276	4	6	0.35	0.62	GQ480185
		R:GTAAGTTCCCGCTTGCTC	55						
PAC62	$(CA)_7$	F:ATCTCACTATCACCCAAACG	55	219	5	7	0.59	0.78	GQ480186
		R:CCATAAGGGTGATGATTGTG	55						
PAC63	$(TG)_5$	F:GTGTGATTGATGCTTGGTTC	56	286	4	6	0.36	0.51	GQ480187
		R:AATATCTCACAAACACTCAGGT	53						
PAC64	$(GT)_{15}$	F:CTGAGGACATTTTTTGTTTGAT	52	327	4	7	0.41	0.62	GQ480188
		R:AACCCTCTTCTGTTACACGAT	56						
PAC65	$(AG)_{20}$	F:TAGTGAGGTCTGAATAGTCTGG	58	298	5	7	0.31	0.40	GQ480189
		R:GCTAAAATAAACACGGCATAAG	54						
PAC66	$(GT)_{15}$	F:TGAGGACATTTTTTGTTTGA	49	278	3	3	0.41	0.53	GQ480190
		R:TGAAACCCAAAACCCTCT	52						
PAC67	$(GGT)_4$	F:TGAGTAATCACAGGCGGTAG	57	327	5	7	0.52	0.72	GQ480191
		R:TTCGGAAACAATGAAACAGG	53						
PAC78	$(CA)_6$	F:CATCTTCACTACTATCCAGGTC	56	324	4	4	0.31	0.52	GQ480192
		R:TTACCATAAGGATGATGATTCT	54						
PSMP1	$(CTCAA)_3$	F:AAATCAAGAATGAGACACGG	55	243	5	5	0.40	0.51	DQ479363.1
		R:AGAAGAAGAGGGTAAATAAAG	58						
PSMP2	$(ATTT)_7$	F:GACTATTTTGCCCCAGACAT	52	357	3	4	0.38	0.47	AY016279.1
		R:AAGATACAAGCAGTTCACGC	56						
PSMP3	$(ATTT)_5$	F:GTCATAAGCGTGTTCCCAGC	54	329	5	5	0.35	0.42	AY016281.1
		R:AATCAAAGACGGTTCGGCAG	57						
PSMP7	$(AG)_8$	F:CATTTCCCACTTCCAACTCT	58	372	4	7	0.36	0.56	AY016265.1
		R:GGCAACACGCTTATGACACA	53						

Microsatellite loci were isolated from widely cultivated tree peony genogype Luoyanghong using *FIASCO* method (Zane *et al.* 2002). A modified version of the cetyltrimethyl ammonium bromide (CTAB) method was used to extract genomic DNA (Han *et al.* 2008). An enriched and partial genomic library was constructed following the FIASCO protocol (Zane *et al.* 2002). Enrichment was performed using the *Streptavidin MagneSphere Paramagnetic Particles* kit (*Promega*, Madison, USA) with biotinylated probes Bio-$(GA)_{15}$ and Bio-$(AC)_{15}$. Recovered DNA was amplified *via* polymerase chain reaction (PCR) using adapter primer. The amplified products were purified with *EZ-10* spin column DNA gel extraction kit (*Sangon Biological Engineering Technology and Services Co*., Shanghai, China), then ligated into the pMD-18T vector (*TaKaRa*, Dalian, China). The ligation products were transformed into DH5α competent cell and positive clones were sequenced by *Sun-Biotech Co*. (Beijing, China). Positive clones were selected randomly to be sequenced and specific primers were designed to flank microsatellite rich regions.

Then, 53 sequences were obtained from 59 randomly selected clones, which were sequenced by *Beijing Sunbiotech Limited Company*. Among those, 30 positive clones were shown to contain SSR motifs when screened with SSRprimer software. Twenty-six primer pairs were designed using *Primer Premier 5.0* software (http:// www.premierbiosoft.com/ primerdesign/). Microsatellite-PCR was conducted with a PCR mixture containing 30 ng of tree peony DNA extracted from leaves, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 U *Taq* DNA polymerase (*TaKaRa*), 0.3 μM each primer and water to a final volume of 20 cm^3. Cycling conditions were: denaturation step at 94 °C for 4 min followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 10 min. PCR products were resolved on 6 % denaturing polyacrylamide gels and visualized by silver staining with *Silver Sequence* DNA sequencing system (*Promega*).

All 26 primers showed high polymorphisms (Table 1). The observed number of alleles ranged between 3 and 7. The observed and expected heterozygosities for each locus were calculated using *GENALEX 6.2* (Peakall and Smouse 2006). Expected (H_e) and observed (H_o) heterozygosity at each locus ranged from 0.42 to 0.78 and 0.28 to 0.59, respectively (Table 1). Tests for linkage disequilibrium and estimate deviations from Hardy-Weinberg equilibrium (HWE) within one population were performed using *Arlequin version 3.1* (Excoffier *et al.* 2005). No significant deviation from Hardy-Weinberg equilibrium after Bonferroni sequential correction for multiple comparisons was observed. No significant linkage disequilibrium was observed between any pair of loci ($P < 0.05$ for all comparisons). The obtained high levels of polymorphism primers combined with which were isolated by Wang *et al.* (2009) will be useful for investigating genetic diversity and studies of population genetic structure in tree peony.

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