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中国特有种爆杖花的微卫星分子标记开发与评价*

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摘要: 爆杖花 (*Rhododendron spinuliferum*) 是中国西南地区特有的观赏和药用植物。为了研究爆杖花和碎米花之间的杂交物种形成过程, 本研究利用 FIASCO 方法对爆杖花进行微卫星引物开发, 从 100 对引物中筛选出 28 个微卫星标记, 其中 22 个为多态。利用爆杖花两个居群共 24 个个体对 22 个多态性位点进行分析, 结果显示: 每个位点具有 2~5 个等位基因, 平均为 3.4 个, 其观测杂合度和期望杂合度分别为 0.083~0.792 和 0.153~0.744。对筛出的 28 个微卫星标记在碎米花的两个自然居群中也做了检测, 结果显示: 有 22 个微卫星标记成功扩增, 其中 20 个有多态性; 每个多态位点有 2~6 个等位基因, 平均为 3.2 个, 其观测杂合度和期望杂合度分别为 0.000~0.833 和 0.117~0.736。开发的微卫星标记可用于爆杖花及其近缘物种的居群遗传学分析和杂交物种形成研究。

关键词: 微卫星标记; 爆杖花; 碎米花; FIASCO; 多态性
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Isolation and Characterization of Microsatellite Markers for the Chinese Endemic Species *Rhododendron spinuliferum* (Ericaceae)

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Abstract: *Rhododendron spinuliferum* (Ericaceae) is an ornamental and medicinal plant endemic to southwest China. In order to study hybridization between *R. spinuliferum* and *R. spiciferum*, a FIASCO (Fast Isolation by AFLP of Sequences Containing Repeats) method was used to develop microsatellite markers in *R. spinuliferum*. A total of 28 microsatellite markers were isolated from 100 SSR primer pairs, of which 22 were polymorphic. Polymorphism of the 22 polymorphic loci was assessed separately in 24 individuals collected from two wild populations. The number of alleles per locus ranged from 2 to 5, with an average of 3.4, while observed (H_O) and expected (H_E) heterozygosities varied from 0.083 to 0.792 and from 0.153 to 0.744, respectively. The same 28 microsatellite markers were also tested in two wild populations (12 individuals from each) of *R. spiciferum*. Twenty two of the markers were successfully amplified, of which 20 were polymorphic. Estimates of diversity in two natural populations of *R. spiciferum* based on the 20 polymorphic markers revealed that number of alleles per locus ranged from 2 to 6, with a mean of 3.2, while observed (H_O) and expected (H_E) heterozygosities ranged from 0.000 to 0.833 and from 0.117 to

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0.736, respectively. These newly developed microsatellite markers will be used in future studies of hybridization and the population genetics of *R. spinuliferum* and its closely related species.

Key words: Microsatellite markers; *Rhododendron spinuliferum*; *Rhododendron spiciferum*; FIASCO; Polymorphism

Rhododendron L. is the largest genus in Ericaceae including about 1 025 species, distributed from the northern temperate zones, throughout tropical southeastern Asia to northeastern Australia (Chamberlain *et al.*, 1996). There are 571 species in China, of which 405 are endemic (Fang *et al.*, 2005). There is a long horticultural history of *Rhododendron*, and lots of species in this genus have been used as ornamental plants (Yang *et al.*, 1999). *Rhododendron spinuliferum* Franch. is one of the important ornamental species, which is endemic to southwest China. The dried stems, leaves and flowers of *R. spinuliferum* can be used as Chinese folk medicine for eliminating phlegm, diminishing inflammation, relieving cough and asthma (Chen *et al.*, 1996).

Natural hybridization has been recognized as an important factor of speciation and diversification within *Rhododendron* (Milne *et al.*, 2010). Numerous instances of natural hybridization have been reported in *Rhododendron* in previous studies (Kron *et al.*, 1993; Zhang *et al.*, 2007; Milne and Abbott, 2008; Ma *et al.*, 2010; Zha *et al.*, 2010). *Rhododendron* ×*duclouxii* is an inferred natural hybrid species between *R. spinuliferum* and *R. spiciferum* Franch. based on morphological characters (Handel-Mazzetti, 1936). This had been confirmed by molecular sequence data recently (Yan *et al.*, 2013). To further study the extent of hybridization between *R. spinuliferum* and *R. spiciferum*, codominant microsatellite markers will be used. In this study, we tried to isolate and characterize suitable microsatellite markers from *R. spinuliferum*, and test their feasibility in *R. spiciferum* samples.

1 Materials and methods

1.1 Materials

Leaf samples of *R. spinuliferum* used to develop microsatellite markers were collected from two natural populations in Yunnan province, China. Total of 24 individuals from two *R. spinuliferum* populations (12 individuals from each) were selected to assess polymorphisms of the developed microsatellite markers (Table 1). The feasibility of the developed microsatellite markers was also assessed in 24 individuals from two *R. spiciferum* natural populations (12 individuals from each) (Table 1). Voucher specimens were deposited in the herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences (KUN).

1.2 Methods

1.2.1 *DNA extraction*

Total genomic DNA was extracted from silica-gel-dried leaves using an improved CTAB (cetyltrimethyl ammonium bromide) method (Liu and Gao, 2011).

Table 1 Details of materials information used in this study

Taxon	Locality	Gepgraphic	Altitude/m	Collection number
R. spinuliferum	Kunming, Panlong area, Shuanglong town	N 25.10° E 102.80°	2070	YLJ-12008, YLJ-12009, YLJ-120010, YLJ-12011, YLJ-12012, YLJ-12013, YLJ-12014, YLJ-12015, YLJ-12016, YLJ-12017, YLJ-12023, YLJ-12031
R. spinuliferum	Chuxiong, Shuangbai county, Tuodian town	N 24.68° E 101.66°	1775	YLJ-12802, YLJ-12803, YLJ-12804, YLJ-12805, YLJ-12806, YLJ-12807, YLJ-12808, YLJ-12809, YLJ-12810, YLJ-12811, YLJ-12813, YLJ-12817
R. spiciferum	Kunming, Panlong area, Shuanglong town	N 25.10° E 102.80°	2070	YLJ-12001, YLJ-12018, YLJ-12019, YLJ-12020, YLJ-12025, YLJ-12027, YLJ-12029, YLJ-12030, YLJ-12036, YLJ-12037, YLJ-12043, YLJ-12051
R. spiciferum	Yuxi, Eshan county, Gaoping town	N 24.22° E 102.32°	1830	YLJ-121089, YLJ-121093, YLJ-121097, YLJ-121111, YLJ-121120, YLJ-121122, YLJ-121123, YLJ-121124, YLJ-121126, YLJ-121127, YLJ-121128, YLJ-121130

1.2.2 *Microsatellite loci isolation, PCR amplification and polymorphism assessment*

The microsatellite loci were isolated based on the FIASCO protocol (Zane *et al.*, 2002). About 300-500 ng genomic DNA was digested with *Mse*I (New England Biolabs, Beverly, Massachusetts, USA), and the digested DNA fragments were ligated to an *Mse*I AFLP adaptor pair (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′) at 37 ℃ for 2 h with T4 DNA ligase (Fermentas, Burlington, Ontario, Canada). Total of 5 μL of a diluted digestion-ligation mixture (1∶10) was used for amplification reactions with the adaptor-specific primers *Mse*I-N (5′-GATGAGTCCTGAGTAAN-3′), with the following cycle program: 95 ℃ for 3 min, 30 cycles of 94 ℃ for 45 s, 50 ℃ for 60 s, 72 ℃ for 60 s, and a final extension step of 7 min at 72 ℃. The amplified fragments (200-800 bp) were enriched for microsatellite repeats by magnetic bead selection with 5′-biotinylated $(AC)_{15}$ and $(AG)_{15}$. These enriched fragments were amplified again with the *Mse*I-N primers. The PCR products were purified using an EZNA Gel Extraction Kit (Omega Bio-Tek, Guangzhou, China). The purified PCR products with enriched microsatellite repeats were ligated into the pGEM-T vector (Promega, Madison, Wisconsin, USA) and transformed into DH5α cells (TaKaRa, Dalian, China). Identification of recombinant clones was performed in a blue/white selection assay. Positive clones were then tested for microsatellite inserts by PCR with $(AC)_{10}$/$(AG)_{10}$ and T7/Sp6 primers, respectively. Clones with positive inserts and appropriate size (300-700 bp) were then sequenced. The sequences of which contain microsatellite repeats (SSRs), and with sufficient flanking regions were then used for designing locus-specific primers with the program Oligo 6.0 (Offerman and Rychlik, 2003).

The polymorphisms of all microsatellite loci were then assessed in 24 individuals from two natural populations (12 individuals from each) of *R. spinuliferum* (Table 1). PCR reactions were performed in 20 μL volumes containing 50-100 ng genomic DNA, 0.6 μM of each primer, 7.5 μL 2× *Taq* PCR MasterMix (containing 0.1 U *Taq* polymerase/μL, 0.5 mM dNTP each, 20 mM Tris-HCl (pH=8.3), 100 mM KCl, 3 mM $MgCl_2$ (Tiangen, Beijin, China). The PCR amplifications were conducted under the following conditions: 95 ℃ for 3 min followed by 32 cycles at 94 ℃ for 30 s, an annealing temperature optimized specifically for each primer pair (Table 2) for 45 s, 72 ℃ for 60 s, and a final extension step at 72 ℃ for 7 min. The amplified fragments were separated on 8% polyacrylamide denaturing gels with a 20 bp molecular size standard ladder (Fermentas, Burlington, Ontario, Canada) and visualized by silver staining. The polymorphic information content was calculated by PIC Calculator. Standard genetic diversity parameters and deviations from the Hardy-Weinberg equilibrium were estimated in GENEPOP version 4.0.10 (Rousset, 2008) for all polymorphic loci. Estimation for linkage disequilibrium between pairs of loci was performed also in GENEPOP version 4.0.10.

2 Results and discussion

A total of 294 clones with positive inserts and appropriate size were sequenced. Among these sequences, 217 (73.8%) sequences were found to contain microsatellite repeats (SSRs), and 133 of these sequences with sufficient flanking regions were suitable for designing locus-specific primers. Finally, total of 100 primer sets were designed for developing microsatellite loci.

The evaluation criteria for the amplification success rate of the loci followed Gao *et al.* (2012). Of the 100 primer pairs tested, 28 loci were successfully amplified, of which 22 showed polymorphisms, and six were monomorphic (Table 2). Sequences of these primers were deposited in GenBank under the accession numbers KC155596 to KC155623. For these polymorphic primers, the number of alleles per locus (A) ranged from 2 to 5, with an average of 3.4, and the values for the observed (H_O) and expected (H_E) heterozygosities ranged from 0.083 to 0.792 and from 0.153 to 0.744, respectively. Five of the

22 polymorphic microsatellite loci deviated from Hardy-Weinberg equilibrium ($P<0.01$) (Table 3), likely due to the presence of null alleles or few tested samples included. There was no significant genotypic linkage disequilibrium (LD) between any pair of loci at $P<0.001$.

Table 2 Characteristics of 28 microsatellite loci developed in *R. spinuliferum*

Locus	Primer sequence (5′-3′)	Repeat motif	Size range/bp	Ta/℃	GenBank accession No.
Polymorphic microsatellites					
Rh003*	F: TCTTCGTCTCCCTCTATCTTT R: AACACACACAGACCTCAAATC	(TC)8	152-176	58	KC155596
Rh005*	F: ATCATTGCTTCTTTTTCCCT R: TCCACCCTCTGTCTCACTCT	(AG)12	164-182	55	KC155597
Rh008*	F: TTGGAGTGAGAACAGAGAGG R: TAATAGGCAGCATCTCCCAT	(AG)14	202-234	55	KC155598
Rh009*	F: GGTAGCCACACTGTTGAAAT R: CTTCCCCTCCATCTTGTTCT	(AG)8	216-230	54	KC155599
Rh017*	F: TTTGGCTCATCGCTTTTAGT R: GAGAGCATCCAAGTCCCTAT	(TC)10	151-175	54	KC155600
Rh020*	F: GCATCTCAAGAACACAATA R: TCAAGAAGGTCCTCCCAGTC	(AG)9	109-143	51	KC155601
Rh031*	F: GAGGAGAGAAAAGGACAAG R: AGTCTTCTTCCTTACCAACG	(AG)14	231-237	49	KC155603
Rh032*	F: GGGCAAACATTCATACATAA R: AGGCAGGCAGGCACCAGAAG	(TC)16	296-308	59	KC155604
Rh034*	F: CAAAAAACACACCGCAGACG R: TGATGGGTGGATGGATAAT	(AG)9	193-203	52	KC155605
Rh037	F: CCTGGGCAAGAGAGAAAACT R: ACAGCGATGGCGATTTGAAC	(AG)8…(AG)11	279-287	55	KC155606
Rh039*	F: TCCTAATCCCTCCATCTCCC R: GCCGTTCCATACAGTACCAA	(TC)10	156-168	57	KC155607
Rh041*	F: CGATTGCCATTTGCCACTACCT R: CCACAACTCCGCTGCTACTG	(TC)7	148-178	55	KC155608
Rh043*	F: AGTTCCCCAAATCTCTTCTC R: TCATTTTCTTTTCTCTGCCT	(AG)23	149-175	53	KC155610
Rh054	F: TGTAGCAAACCCATCTCACC R: TCACCTGGGCATAACTAATC	(TC)8	261-275	58	KC155611
Rh058*	F: GATATGGACTCCGACAAGGT R: GGCGAGATCGTGGAGAAAAT	(TC)9	174-180	58	KC155612
Rh060*	F: AAGAGATTGGAAGGGTTGAT R: TCATAGTGTGGCAAAACGAC	(AG)7…(AG)7	166-172	54	KC155613
Rh063	F: TGACGACATGGGACTTTAGA R: ACCCTTTCTTCATCTTCCAG	(TC)20	164-172	52	KC155614
Rh065	F: TAAAAAAATGGGGCTAAAGT R: GACATTGACGCAGCCGAACC	(AG)16	261-283	50	KC155616
Rh072*	F: GCTCTACCCTTATCATTTTA R: AAGACGGACGAAACACATC	(TC)25	169-181	57	KC155617
Rh076*	F: ATACACCACCATTCATACGC R: TAGAGAGTGGGGTTGATTAG	(AG)17G(AG)6…(AG)8	258-310	20	KC155618
Rh078*	F: CAATGATGTGAAAGCCCTGG R: AGGATTCCAATTAGTAAACG	(TG)8	284-300	50	KC155619

Table 2 continued

Locus	Primer sequence (5′-3′)	Repeat motif	Size range/bp	Ta/℃	GenBank accession No.
Rh086*	F: ATCACCCAAGCAATAGTCTG R: ATTTTCCACACGATACAGGC	(TC) 9… (TG) 8	269-281	57	KC155620
Monomorphic microsatellites					
Rh023*	F: CTACCATCAACATCACACTG R: AGTAAAAAGAGAAGGGGAGT	(TC)8	131	52	KC155602
Rh042*	F: CACAAGTGTTCCAAGATTCG R: GACGGGAGTTATCGGTGAAG	(TC)7C(TC)9	165	55	KC155609
Rh064*	F: GATGGTAGTTTCAACGCAAG R: ACTCCTTTCTTTTCTCACCT	(AG)9	193	52	KC155615
Rh087	F: AGAATAGAAGGTTGAAGGGT R: AAGGCTTGAATGAGGTTGAT	(TC)13	217	52	KC155621
Rh096*	F: CCCTCCTCTCTCAACAAAAG R: TCAGAGTTGTTCGGTGTGTG	(TC)10	157	54	KC155622
Rh098	F: AAACCCCATTACAGTAGATT R: ACTGGACCCTTGAAACCTAAC	(AG)9	189	50	KC155623

Note: Ta, PCR annealing temperature; *, successful amplification in *R. spiciferum*

Table 3　Results of the polymorphic microsatellite loci evaluated in two wild populations (12 individuals from each) of *R. spinuliferum* and *R. spicifeum* respectively

Locus	*R. spinuliferum*				*R. spicifeum*			
	N_A	H_O	H_E	P_{HW}	N_A	H_O	H_E	P_{HW}
Rh003	4.000	0.739	0.616	0.113	3.000	0.833	0.542	0.005*
Rh005	4.000	0.375	0.318	1.000	2.000	0.250	0.278	0.502
Rh008	4.000	0.417	0.357	1.000	4.000	0.667	0.587	0.142
Rh009	2.000	0.083	0.153	0.128	2.000	0.545	0.496	1.000
Rh017	5.000	0.750	0.724	0.254	2.000	0.500	0.469	1.000
Rh020	4.000	0.792	0.744	0.090	2.000	0.542	0.430	0.355
Rh023					2.000	0.042	0.117	0.063
Rh031	4.000	0.333	0.510	0.011	4.000	0.500	0.688	0.148
Rh032	3.000	0.565	0.638	0.336	4.000	0.333	0.641	0.001*
Rh034	3.000	0.250	0.227	1.000	4.000	0.708	0.736	0.023
Rh037	4.000	0.227	0.714	0.000*				
Rh039	4.000	0.667	0.643	0.565	3.000	0.542	0.594	0.477
Rh041	4.000	0.583	0.513	1.000	3.000	0.500	0.492	0.301
Rh043	2.000	0.458	0.430	1.000	3.000	0.250	0.624	0.000*
Rh054	2.000	0.174	0.476	0.002*				
Rh058	4.000	0.417	0.506	0.091	4.000	0.792	0.711	0.104
Rh060	3.000	0.250	0.473	0.001*	3.000	0.333	0.645	0.008*
Rh063	3.000	0.625	0.555	1.000				
Rh065	3.000	0.708	0.661	0.670				
Rh072	3.000	0.208	0.659	0.000*	5.000	0.708	0.728	0.523
Rh076	4.000	0.375	0.704	0.000*	6.000	0.333	0.538	0.016
Rh078	3.000	0.333	0.351	0.157	3.000	0.333	0.586	0.015
Rh086	3.000	0.208	0.320	0.056	2.000	0.000	0.287	0.000*
Rh096					2.000	0.208	0.492	0.006*

Note: N_A, number of alleles revealed; H_O, observed heterozygosity; H_E, expected heterozygosity; *, polymorphic microsatellite loci deviating from Hardy-Weinberg equilibrium ($P<0.01$).

The 28 microsatellite markers were also tested in *R. spiciferum* using the same PCR conditions as in *R. spinuliferum*. Of the 28 loci tested, 22 SSR markers were amplified successfully, of which 20 loci showed polymorphisms and two loci were monomorphic (Rh042 and Rh064) in *R. spiciferum* (Table 2 & 3). The two monomorphic microsatellite markers (Rh023 and Rh096) in *R. spinuliferum* showed polymorphism in *R. spiciferum* (Table 2 & 3). For the 20 polymorphic markers of *R. spiciferum*, the number of alleles per locus ranged from 2 to 6, with a mean of 3.2. The observed (H_O) and expected (H_E) heterozygosity ranged from 0.000 to 0.833 and from 0.117 to 0.736, respectively. Six of the 20 polymorphic microsatellite loci deviated from the Hardy-Weinberg equilibrium ($P<0.01$) (Table 3).

In summary, of the 28 microsatellite markers firstly developed in *R. spinuliferum*, most worked in *R. spiciferum* (79%). Thus, these codominant microsatellite markers developed in this study will be very useful to investigate the hybrid speciation scenario between *R. spinuliferum* and *R. spiciferum*, and also be useful to assess the genetic diversity and population structure of *R. spinuliferum* and other closely related species.

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