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Polymorphic microsatellite loci for the genetic analysis of *Lycoris radiata* (Amaryllidaceae) and cross-amplification in other congeneric species

S.Q. Xuan[1], J.Y. Zheng[1], H. Wang[1,2], P. Gao[1], S.B. Zhou[1,2], K. Liu[1,2] and G.-P. Zhu[1,2]

[1]Key Laboratory of Molecular Evolution and Biodiversity,
Institute of Molecular Biology and Biotechnology,
Anhui Normal University, Wuhu, P.R. China
[2]Anhui Provincial Key Laboratory of the Conservation and Exploitation of Biological Resources, Anhui Normal University, Wuhu, P.R. China

Corresponding author: G.-P. Zhu
E-mail: gpz1996@yahoo.com

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ABSTRACT. *Lycoris radiata* is a perennial herb that has been used in traditional Chinese medicine for a long time and has two main medicinal components in its bulb, lycorine and galanthamine. However, the original microsatellite loci have not been developed for any species of *Lycoris*. Total genomic DNA was extracted from fresh bulbs using a modified CTAB protocol. We isolated 10 microsatellite loci from 21 *L. radiata* individuals of a natural population from Yellow Mountain in Anhui Province, China. The number of alleles ranged from two to nine. The observed and expected heterozygosities ranged from 0.238 to 0.952 and from 0.455 to 0.784, respectively. One locus significantly deviated from Hardy-Weinberg equilibrium and no significant linkage disequilibrium was found between pairs of loci. Cross-species amplification of these microsatellite loci was characterized in additional five species (*L. sprengeri*, *L. anhuiensis*, *L. albiflora*, *L. longituba*, and

L. chinensis) of *Lycoris*. The results suggest that these microsatellite markers would contribute to the population genetic studies of *L. radiata* and other related species.

Key words: *Lycoris radiata*; Microsatellite; Molecular marker; Cross-amplification

INTRODUCTION

Lycoris radiata is a perennial herb grown from small bulbs. The plant prefers shady and moist areas and is mainly distributed in the middle and lower reaches of the Yangtze River in China and other countries in Southeast Asia (Ji and Meerow, 2000). Their bulbs have been used in traditional Chinese medicine for a long time. There are two main medicinal components in the bulbs, lycorine and galanthamine. Galanthamine has been widely used in medicine as a strong reversible inhibitor of cholinesterase to increase acetylcholine sensitivity (Novikova1 and Tulaganov, 2002). It has also been used in the treatment of glaucoma (Harvey, 1995) and Alzheimer's disease (Harvey, 1995; Bores et al., 1996). Furthermore, *L. radiata* is of great interest to horticulturists due to its scarlet flowers in autumn and green leaves all winter long. The morphology of *L. radiata* has been extensively studied, including pollen (Zhou et al., 2005) and leaf morphology (Zhou et al., 2007a) and karyotype analysis (Zhou et al., 2007b). However, studies on molecular phylogeny and population genetics are very scarce. Microsatellites are widely distributed across genomes and inherit in a codominant Mendelian manner and display high levels of polymorphism **(**Li et al., 2002; Menezes et al., 2009). They have been developed into one of the most popular genetic markers used in parentage, genomic mapping, evolutionary population genetics, conservation biology, and other studies (Rao et al., 2000; Ellegren, 2004; Jarvis et al., 2008; Peng et al., 2009). However, the original microsatellite loci have not been developed for any species of *Lycoris*. In this study, 10 polymorphic microsatellite DNA markers were developed from *L. radiata* using a modified biotin-capture method (Bloor et al., 2001).

MATERIAL AND METHODS

Total genomic DNA was extracted from fresh bulbs using a modified cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle and Doyle, 1987; Varma et al., 2007). The enriched partial genomic library for the repeat motif $(CT)_{15}$ was constructed using the individual DNA and the microsatellite-enriched DNA fragments were captured by a modified biotin-capture method (Bloor et al., 2001). Briefly, genomic DNA was digested with *Sau*3AI (TaKaRa). DNA fragments between 300 and 900 bp were isolated on 1.2% agarose gel and ligated to the *Sau*3AI adaptors oligoA (5'-GGCCAGAGACCCCAAGCTTCG-3') and oligoB (5'PO4-GATCCGAAGCTTGGGGTCTCTGGCC-3') (Bloor et al., 2001). Then, DNA fragments were amplified by polymerase chain reaction (PCR) using oligoA primers. The genomic DNA fragments containing simple-sequence repeats (SSR) were hybridized to the single-stranded 3'-biotinylated $(CT)_{15}$ oligonucleotide probes and captured by streptavidin-coated beads (Promega). Captured fragments were ligated to pMD18-T vector (TaKaRa) and transformed into *Escherichia coli* DH5α competent cells according to the standard protocol. PCRs using oligoA

and oligonucleotides $(CT)_{15}$ as the primers were employed to identify insert-positive clones. The clones that yielded two or more bands contained microsatellite fractions, as shown in Figure 1. Sixty-six positive clones were screened and sequenced using the ABI PRISM 3730 automated DNA sequencer. Forty-five clones were successfully sequenced and 40 sequences contained repeat motifs. Thirty-three pairs of primers were designed using the PRIMER PREMIER 5.0 software (http://www.premierbiosoft.com/) and synthesized.

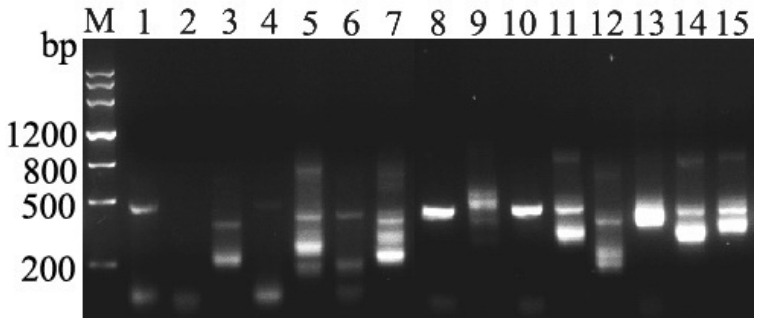

Figure 1. Agarose gel showing the representative identification of captured fragment insert-positive clones. *Lane M* = molecular marker; *lanes 1-15* = PCRs to identify insert-positive clones using oligoA and oligonucleotides $(CT)_{15}$ as the primers.

Polymorphism at each locus was determined using 21 individuals from a natural population from Yellow Mountain (Mt. Huangshan) in Anhui Province. PCR amplifications (15 µL) contained 6.25 µL GoTaq® Green Master Mix (Promega, China), 0.5-1.0 µmol of each primer and 10-20 ng DNA, and were performed in an iCycler thermocycler (Bio-Rad). The cycling parameters were 4 min at 94°C followed by 35 cycles of 45 s at 94°C, 35 s at the optimized annealing temperature (Table 1) and 30 s at 72°C. The final extension was 8 min at 72°C. The PCR products were separated on 8% denaturing polyacrylamide gels (acrylamide:bisacrylamide, 19:1) using a LI-COR 4200 automated DNA sequencer and analyzed using the LI-COR SAGA^{GT} software. GENEPOP version 4.0 (Raymond and Rousset, 1995) was used to calculate the number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities, Hardy-Weinberg equilibrium expectation (HWE), and linkage disequilibrium. All results for multiple tests were corrected using Bonferroni's correction (Rice, 1989).

RESULTS AND DISCUSSION

Ten polymorphic microsatellite loci from the microsatellite-enriched genomic library were newly developed across 21 *L. radiata* individuals. Details are summarized in Table 1. The 10 sequences containing microsatellite loci were deposited in GenBank (HQ697319-HQ697328), and no similarity was found between the 10 microsatellites and the sequences published in GenBank. The N_A per locus ranged from 2 to 9. The H_O and H_E ranged from 0.238 to 0.952 and from 0.455 to 0.784, respectively. The remaining 23 loci were monomorphic and failed to amplify. Nine loci conformed to HWE and one locus (Lyra-7) significantly deviated from HWE expectations in the sampled population after Bonferroni's correction (adjusted P value = 0.0044). Lyra-7 deviated from HWE, which could be due to the presence of null alleles

that was confirmed by the analyses using the MICRO-CHECKER software (Van Oosterhout et al., 2004) (Bonferroni's correction). None of the loci showed significant linkage disequilibrium.

Table 1. Characteristics of 10 polymorphic microsatellite loci isolated from *Lycoris radiata*.

Locus	Repeat motif	Primer sequence (5'-3')	Ta (°C)	Allele size (bp)	N_A	H_O	H_E	GenBank accession No.
Lyra-1	$(CT)_{16}$	F: TCTGTTCACCTTTACCCTCTC R: CAGCCTCAACTCCAATCTCG	57	131-197	6	0.524	0.657	HQ697319
Lyra-2	$(TC)_{35}$	F: CCATCGCCGCTACTATCAC R: AAAGGAGAAGAGGGTATCAAC	56	180-232	9	0.762	0.784	HQ697320
Lyra-3	$(CT)_{24}$	F: CCAATGTTCACTACCACCCC R: GAAGGTTTTGGTTTCAGGAT	53	200-300	4	0.571	0.672	HQ697321
Lyra-4	$(TC)_{31}$	F: CCTTGCTCACGACTCCTTTA R: CCAATGCTGTTGCCACCTTC	60	306-350	5	0.905	0.740	HQ697322
Lyra-5	$(TC)_{12}C(CT)_7$	F: CCTTCTTTGGCTACTCACAT R: CAGTCAGTCCATCCTCTTGC	59	265-297	4	0.952	0.700	HQ697323
Lyra-6	$(TC)_{19}$	F: CATCCCCTCCCCGAACTAT R: GCTTCTCACCGCCGCTAAT	59	288-316	2	0.286	0.455	HQ697324
Lyra-7*	$(AG)_{19}$	F: GGGTGATGAAATGAACTG R: ACTCCTATTGCCACCCTC	53	224-262	3	0.238	0.528	HQ697325
Lyra-8	$(AG)_7N(GA)_{19}$	F: CAGTCGCTAAACTAAGCCCTAT R: CCCACAACTTGCCTTCCTCT	59	225-287	6	0.619	0.740	HQ697326
Lyra-9	$(GA)_{24}$	F: AACTCCAACCACCCTTTA R: ACTTGCCTTCCTCTTTCT	54	257-297	5	0.524	0.698	HQ697327
Lyra-10	$(CT)_{22}$	F: TCGCTCAACTTACCTCTA R: TGATTCCTCCATGCTTCT	51	215-251	3	0.524	0.463	HQ697328

Ta = optimized annealing temperature; N_A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity. *Indicates significant deviation from HWE after Bonferroni's correction ($P < 0.05$).

Cross-species amplification of 10 microsatellite markers was carried out in five congeneric species: *L. sprengeri*, *L. anhuiensis*, *L. albiflora*, *L. longituba*, and *L. chinensis*. The same PCR conditions were used as described above except that the annealing temperature was re-optimized at each locus. Five individuals from each species were screened at 10 loci. Results are summarized in Table 2. Two loci (Lyra-5 and Lyra-6) were amplified successfully in all species, two loci (Lyra-1 and Lyra-2) were amplified in four species except *L. sprengeri*, and nine loci (except Lyra-10) were amplified in *L. albiflora*. These results suggest that the identified microsatellite markers will be useful in genetic studies of *L. radiata* and other species of *Lycoris*.

Table 2. Cross-species amplification of 10 microsatellite loci from *Lycoris* species.

Locus	*L. sprengeri*	*L. anhuiensis*	*L. albiflora*	*L. longituba*	*L. chinensis*
Lyra-1	-	167-195	153-207	153-207	147-200
Lyra-2	-	200	168-200	156-192	152-192
Lyra-3	-	-	216-262	331	-
Lyra-4	-	-	285	-	-
Lyra-5	287	258-300	225-287	215-287	240-300
Lyra-6	315	320-350	315-345	348	348
Lyra-7	-	-	350-300	-	-
Lyra-8	225	-	225	-	-
Lyra-9	-	-	262	-	-
Lyra-10	225	225	-	-	235

Minus sign denotes no visible PCR product.

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