GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Isolation and characterization of novel EST-SSRs in the showy dendrobium, *Dendrobium nobile* (Orchidaceae)

J.J. Lu*, J.Y. Kang*, S.R. Ye and H.Z. Wang

Zhejiang Provincial Key Laboratory for Genetic Improvement and Quality Control of Medicinal Plants, Hangzhou Normal University, Hangzhou, China

*These authors contributed equally to this study.
Corresponding author: H.Z. Wang
E-mail: whz62@163.com

Genet. Mol. Res. 13 (1): 986-991 (2014)
Received January 4, 2013
Accepted July 17, 2013
Published February 19, 2014
DOI http://dx.doi.org/10.4238/2014.February.19.10

ABSTRACT. Expressed sequence tag (EST)-derived simple sequence repeat (SSR) markers could enrich the current resource of molecular markers. In this study, microsatellite markers were developed for *Dendrobium nobile* Lindl. by mining the ESTs. Twenty-eight EST-SSRs amplified 2 to 6 nucleotide repeats with a mean number of 2.82 alleles per locus. The observed and expected heterozygosities per locus ranged from 0.158 to 0.579 and 0.422 to 0.752, respectively. These novel EST-SSRs enriched the current resource of molecular markers for the *Dendrobium* genus and would facilitate further applications in germplasm appraisal, evolution and genetic diversity studies, genetic mapping, and plant breeding of *D. nobile* and other congeneric species.

Key words: *Dendrobium nobile*; Expressed sequence tag; Simple sequence repeat marker

INTRODUCTION

Dendrobium nobile Lindl. is a perennial herb in the family Orchidaceae, which is famous for both horticultural and medical value all around the world. As one of the most widespread ornamental orchids, this species retains characteristics of different created varieties that bloom in different colors (Wang et al., 1999); as one of the 5 fundamental medical *Dendrobium* species, it was listed in the Chinese Pharmacopoeia (Chinese Pharmacopoeia Editorial Committee, 2000) and used as traditional Chinese medicine for centuries (Bulpitt et al., 2007). Unfortunately, the wide germplasm resources of this species are now under first-grade state protection in China because of overexploitation and habitat deterioration.

Currently, it is necessary to develop more molecular markers such as microsatellite markers, which are dispersed extensively within the genome and are highly polymorphic, to overcome the limited availability of genetic marker resources that hinder genetic research in *D. nobile*. However, as a non-model plant that lacks a sequenced genome, thus far only over a hundred *Dendrobium* simple sequence repeat (SSR) markers have been reported (Gu et al., 2007; Xie et al., 2010; Lu et al., 2012a,b). Here, we present the development of novel polymorphic expressed sequence tag (EST)-SSRs in *D. nobile*, which will be helpful for potential genetic applications.

MATERIAL AND METHODS

Plant materials and DNA extraction

The wild plants of *D. nobile* were collected from Simao (21°77'N, 100°96'E), Yunnan, China, and transplanted to the greenhouse of Hangzhou Normal University (120°19'E, 30°26'N), China. The species identity was verified and confirmed using the specimen of *D. nobile* (Barcoding No. 00293937) that was stored in the herbarium at the Institute of Botany, Chinese Academy of Sciences, Beijing, China (http://www.nhpe.org/pe/00293937).

A modified cetyltrimethylammonium bromide (CTAB) extraction method was used to extract the genomic DNA from fresh leaf tissues (Rogers and Bendich, 1985), and the concentration of each DNA sample was determined by NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA).

Isolation of EST-SSRs, primer design, and marker validation

In this study, a normalized flower organ EST library was constructed for the wild plants of *D. nobile*. The UniVec database (ftp://ftp.ncbi.nih.gov/pub/UniVec/) was used to detect vector and adapter sequences of all of the ESTs; the EST_trimmer.pl script (http://pgrc.ipk-gatersleben.de/misa/download/est_trimmer.pl) was used to remove the polyA/T tails and N characters; following the removal of the redundant sequences by the assembly CAP3 program (Huang and Madan, 1999), a Perl-based script (MISA, http://pgrc.ipk-gatersleben.de/misa/) was used to screen for the presence of microsatellites

and was set to detect tandem repeats of 2-6 nucleotides for at least 5 perfect repeats as core motifs. At last, primers for the candidate SSR loci in ESTs were designed using Primer 3.0 (Rozen and Skaletsky, 2000) with the following settings: primer length, 20 ± 2 nucleotides; GC content, 40-60%; optimum annealing temperature, at least 50°C; and PCR product size, 100-300 bp. The primers were used to amplify SSR loci in *D. nobile* genomic DNA, and the amplified bands with bands that were clear and the expected size were sequenced to validate the corresponding repeat motifs. All of the sequences of the developed *D. nobile* SSRs were compared with the *Dendrobium* SSRs that were previously reported (Gu et al., 2007; Xie et al., 2010; Lu et al., 2012, 2013) in order to determine that they were novel.

PCR conditions and data analysis

PCR was performed in a 20-μL reaction mixture that contained 50-100 ng genomic DNA, 1 U *Taq* polymerase with 1X PCR universal buffer, 1.5 μM $MgCl_2$, 200 μM each dNTP (TaKaRa Bio. Inc., Kyoto, Japan), and 0.2 μM of each primer. PCR amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA) with the following program: 95°C for 5 min; 30 cycles of 1 min at 95°C, 1 min at the annealing temperature of each primer pair (Table 1), and 2 min at 72°C; and a final step at 72°C for 5 min. The PCR products were resolved on a 6% (w/v) polyacrylamide denaturing sequencing gel (7 M urea) using a Sequi-Gen GT sequencing cell (Bio-Rad, Hercules, CA, USA). SSR patterns were revealed using silver staining, and the SSR fragment size was evaluated with a 10-bp DNA ladder (Invitrogen, Carlsbad, CA, USA).

The polymorphism levels of each locus were assessed in 24 *D. nobile* individuals from Simao, Yunnan, in the southwestern part of China. The number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities, linkage disequilibrium (LD), and deviations from Hardy-Weinberg equilibrium (HWE) were analyzed using the GENEPOP version 4.0.10 software (Rousset, 2008). CERVUS version 3.0.3 (Kalinowski et al., 2007) was employed to calculate the value of polymorphism information content (PIC).

RESULTS AND DISCUSSION

As shown in Table 1, 28 *D. nobile* EST-SSRs were polymorphic within 24 individuals. The N_A was 2.82, the mean H_O was 0.350 (range: 0.158-0.579), the mean H_E was 0.608 (range: 0.422-0.752), and the mean PIC value was 0.592 (range: 0.411-0.733). No significant LD was detected ($P > 0.01$), and 18 of 28 primer pairs displayed deviation from HWE ($P < 0.05$) in the Simao wild population that we surveyed.

The in-depth investigation of the population dynamics and molecular genetics of *D. nobile* and *Dendrobium* species has been hindered largely as a result of the lack of highly polymorphic codominant markers. These newly developed 28 polymorphic *D. nobile* EST-SSRs represent an additional resource for germplasm appraisal, evolution and genetic diversity studies, and genetic mapping and plant breeding of *D. nobile* and other congeneric species.

Table 1. Characteristics of 28 novel polymorphism SSR markers in *Dendrobium nobile*.

Locus	Accession No.	Primer sequence (5'-3')	Repeat motif	Ta (°C)	Size (bp)	N_A	H_O	H_E	PIC	HWE
DNeSSR5	JX033716	F: GAGAAGAGGAAGAGCAAGAAT R: GCCTAAGAGAAGAAAGTCCAC	$(TCT)_6$	54	164	3	0.211	0.624	0.608	0.0000***
DNeSSR6	JX033717	F: GGTTTGAAATGTTTCTCATCA R: ACTTCTCATTGGTGATCTGC	$(CT)_{13}$	55	149	3	0.316	0.607	0.591	0.0000***
DNeSSR9	JX033720	F: AAGAGATGGGTTCTCTTTCAC R: CAGCTCAAGAACATGACTTTT	$(CTT)_7$	55	142	3	0.211	0.542	0.527	0.0000***
DNeSSR17	JX033728	F: TTAAGGGGCGGAGTAAGT R: GAATGTTAACCTCCAAGCTCT	$(AGA)_8$	55	157	4	0.474	0.694	0.675	0.0038**
DNeSSR24	JX033735	F: GGACGTGTGCAATATCATAAC R: GGTACAGGTGGTTGTTTGAC	$(GTG)_4(TGGCCA)_3$	55	140	4	0.211	0.741	0.721	0.0000***
DNeSSR25	JX033736	F: GCTCAATTGGTGTAAATGGT R: CTAGCTCTGATGAGCTTCTTG	$(TGA)_{10}$	55	132	2	0.263	0.422	0.411	0.0002***
DNeSSR27	JX853729	F:AAAGCGTCAATAAGGGAACT R:AGCAATGGAGCGAAGATAG	$(AAG)_{10}$	244	53	3	0.368	0.664	0.648	0.0006***
DNeSSR37	JX853730	F:ATGGGGAGGGTTGCAAATAT R:ACCGCTGCGTCCAGTAATCA	$(CT)_{26}$	261	60	2	0.211	0.478	0.465	0.0000***
DNeSSR40	JX033738	F: TTCTGCCGGGGTATCTAG R: ATCCATCCGATAAGCACC	$(GCGA)_5(GA)_8$	55	137	3	0.263	0.661	0.644	0.0024**
DNeSSR46	JX033757	F: TGATGGAGTGAGGAGGAACT R: TTGGTGAGAAAGGCATAAAA	$(CAG)_4$	53	169	3	0.579	0.683	0.665	0.2678 n.s.
DNeSSR52	JX033763	F: AACGACGATGATGACGAAGG R: TCAAAGGTTCCGAGCCATAC	$(CCG)_4$	58	183	3	0.368	0.649	0.631	0.0005***
DNeSSR58	JX033769	F: ACTACGCGATCAGGTCTGTG R: CTCTGGTGCTTACTACAATGCT	$(CTT)_7$	55	129	2	0.316	0.512	0.499	0.1645 n.s.
DNeSSR60	JX033771	F: GAGATCGGAAGCCTAGCAGA R: CCCTCGCCATTAAATCTTTG	$(AGA)_4$	57	295	4	0.421	0.735	0.716	0.0062**
DNeSSR74	JX033785	F: CCGCAAAATGGTCAAATAAA R: GCTTGGTAAAATCTGCAATA	$(TA)_5$	53	151	4	0.421	0.752	0.733	0.0000***
DNeSSR85	JX033796	F: TCTGGAGGAAGCGAGAAAGC R: TACAACAGCGTAGCGTCTGC	$(TG)_6(TGA)_4$	58	205	4	0.421	0.750	0.730	0.0011**
DNeSSR86	JX033797	F: AAGCAGCGGGAGGAGCAGAA R: ACATCGCAGTCATCAATGTAA	$(TC)_7$	58	269	3	0.316	0.660	0.643	0.0118*
DNeSSR92	JX033802	F: ACTTCTAGGGTTAGGGTTTGTG R: AAGGTCTTGAAACAGGCATC	$(CGGCAA)_3$	55	261	3	0.368	0.622	0.606	0.0062**
DNeSSR97	JX033807	F: GGCTACTGTTCCCGATGCTC R: GGCTGAATGGATACGGTCTT	$(TTCA)_3$	58	180	2	0.368	0.491	0.478	0.1183 n.s.
DNeSSR99	JX033809	F: GATTTGAGGGATTCGAGATG R: AGTTCATTCAGGGAACCATA	$(TGCT)_3$	53	224	2	0.421	0.512	0.499	0.6445 n.s.

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Table 1. Continued.

Locus	Accession No.	Primer sequence (5'-3')	Repeat motif	Ta (°C)	Size (bp)	N_A	H_O	H_E	PIC	HWE
DNeSSR111	JX033821	F: TGGCACCAACCGTACTAATC R: TCTATCGTCTTCTTCATCTTCG	$(CACCAT)_3$	55	170	3	0.526	0.684	0.666	0.4869 n.s.
DNeSSR112	JX033822	F: ATTTCTTCGATTTCATGTGC R: TTCCTAGAGGTCGGAGTGTC	$(CTT)_4$	53	185	2	0.158	0.514	0.500	0.0037**
DNeSSR114	JX033824	F: TCAACTCCCGAAGAAAAGTC R: GCACATAGTTCCAACAAATAA	$(TA)_7$	55	236	3	0.421	0.664	0.648	0.1030 n.s.
DNeSSR158	JX033868	F: AGTTGCCTGAAGATTTTGCT R: ATCCCTGACAGATAGATGTGCT	$(CT)_{12}$-$(CT)_5$	55	224	2	0.368	0.514	0.500	0.0600 n.s.
DNeSSR164	JX033874	F: CAGGATGCTCTAAGCCAGTA R: AAAGAACAATGAAAGGCAAG	$(TC)_8$	53	152	3	0.421	0.681	0.664	0.0028**
DNeSSR166	JX033876	F: TCCGTGTCCTTGTTTCTTAC R: TCTTCCCTCTTTCCTCTTCT	$(AG)_8$	53	185	2	0.316	0.512	0.499	0.0625 n.s.
DNeSSR170	JX033880	F: AGAAGCTCGCTGTCTCCATC R: TGAAGTTGCTGCTGTTGTTGT	$(GCC)_4$-$(CAA)_4$	57	240	2	0.263	0.508	0.494	0.0597 n.s.
DNeSSR178	JX033888	F: TGGATACAGTTCCCATCACCT R: AACATACTTCCCAATACCAACG	$(CGG)_5$	57	237	2	0.316	0.512	0.499	0.1649 n.s.
DNeSSR180	JX033890	F: CAGGGAGGGGAATGAGGTAT R: AGAAGAGTAGCACAACGAAAA	$(GTGCT)_4$	55	181	3	0.474	0.639	0.622	0.0056**

Ta = annealing temperature; N_A = number of alleles; H_O/H_E = observed and expected heterozygosities; PIC = polymorphic information content; HWE = Hardy-Weinberg equilibrium. *,**, and *** = significant departures from Hardy-Weinberg equilibrium at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. n.s. = not significant.

ACKNOWLEDGMENTS

Research supported in part by the National Natural Science Foundation (#31070298 and #30870180), the Zhejiang Scientific and Technological Program (#2008C12081), the Project of Zhejiang Provincial Key Laboratory for Genetic Improvement and Quality Control of Medicinal Plants, and the Hangzhou Scientific and Technological Program (#20101032B25).

REFERENCES

Bulpitt CJ, Li Y, Bulpitt PF and Wang J (2007). The use of orchids in Chinese medicine. *J. Roy. Soc. Med.* 100: 558-563.

Chinese Pharmacopoeia Editorial Committee (2000). Pharmacopoeia of the People's Republic of China. Chemical Industry Press, Beijing.

Gu S, Ding XY, Wang Y and Zhou Q (2007). Isolation and characterization of microsatellite markers in *Dendrobium officinale*, an endangered herb endemic to China. *Mol. Ecol. Notes* 7: 1166-1168.

Huang X and Madan A (1999). CAP3: A DNA sequence assembly program. *Genome Res.* 9: 868-877.

Kalinowski ST, Taper ML and Marshall TC (2007). Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Mol. Ecol.* 16: 1099-1106.

Lu JJ, Suo NN, Hu X, Wang S, et al. (2012). Development and characterization of 110 novel EST-SSR markers for *Dendrobium officinale* (Orchidaceae). *Am. J. Bot.* 99: e415-e420.

Lu JJ, Gao L, Kang JY and Feng SG (2013). Thirteen novel polymorphic microsatellite markers for endangered Chinese endemic herb *Dendrobium officinale*. *Conserv. Genet. Resour.* 5: 359-361.

Rogers SO and Bendich AJ (1985). Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant-tissues. *Plant Mol. Biol.* 5: 69-76.

Rousset F (2008). GENEPOP'007: a complete re-implementation of the genepop software for Windows and Linux. *Mol. Ecol. Resour.* 8: 103-106.

Rozen S and Skaletsky H (2000). Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* 132: 365-386.

Wang Y, Li ZJ and Peng HM (1999). The *Dendrobium*. Science Press, Beijing.

Xie ML, Hou BW, Han L, Ma YH, et al. (2010). Development of microsatellites of *Dendrobium officinale* and its application in purity identification of germplasm. *Yao Xue Xue Bao* 45: 667-672.