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Applications in Plant Sciences

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Primer Note

Development of transcriptome-derived SSR markers for *Hoya ledongensis* (Apocynaceae) and cross-amplification in a congener[1]

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- *Premise of the study:* To examine patterns of genetic diversity and test possible hybridization events, microsatellite markers were identified and characterized in *Hoya ledongensis* (Apocynaceae), and cross-amplification was tested in a congener, *H. jianfenglingensis*.
- *Methods and Results:* Based on the transcriptome data of *H. ledongensis*, 46 microsatellite primer pairs were randomly selected for initial validation. From these, 28 primer pairs were successfully amplified, 12 of which were polymorphic in 36 individuals across three populations of *H. ledongensis*. The number of alleles per microsatellite locus ranged from two to 11. The observed and expected heterozygosities for the 12 loci ranged from 0.133 to 0.867 and 0.128 to 0.894, respectively. Cross-species amplification was successful for these 12 loci in the congeneric species *H. jianfenglingensis*.
- *Conclusions:* These polymorphic transcriptome-derived simple sequence repeat markers have the potential to be used as multilocus molecular markers to study the population genetics and natural hybridization in species of *Hoya*.

Key words: Apocynaceae; conservation; genetic diversity; *Hoya ledongensis*; transcriptome-derived SSR markers.

Hoya R. Br. (Apocynaceae) is composed of 200–300 species worldwide and is an important epiphyte component of tropical and subtropical forests (Liede and Albers, 1994). It is mainly distributed in the tropical rainforests of Southeast Asia, Australia, and islands of the Indian and Pacific oceans (Wanntorp, 2009). China is one of the main distribution areas of *Hoya*, with 39 species mainly occurring in Yunnan, Guangxi, Guangdong, and Hainan provinces (Li and Jiang, 1977; He et al., 2009a, b, c, 2011a, b, 2012).

In China, many species of this genus occur in a very narrow distribution range. For example, *H. ledongensis* Shao Y. He & P. T. Li is restricted to the central mountain areas of Hainan Province and *H. jianfenglingensis* Shao Y. He & P. T. Li is only known from Jianfengling Nature Reserve, Hainan (He et al., 2011a, b). For the latter species, it is estimated that there are fewer than 60 individuals in its whole range (He et al., 2011a). Therefore, conservation of these lands that provide narrow distribution ranges for these species should be prioritized. Knowledge of genetic diversity and population structure of *Hoya* species can provide important information for their conservation. Furthermore, both of these species occur at high elevations (ca. 1000 m) and are sympatric in the area of the Jianfengling Nature Reserve. *Hoya ledongensis* flowers from May to June, and *H. jianfenglingensis* flowers from May to July (He et al., 2011a, b). The overlap in flowering time between the two species and the insect-pollination mating system for *Hoya* provide an opportunity for interspecific hybridization. Molecular markers can be used to address these evolutionary questions on conservation genetics and natural hybridization in this genus. With advances in high-throughput sequencing technologies, transcriptome-derived simple sequence repeat (SSR) markers can be easily obtained and are also increasingly used in conservation genetic studies (Yu et al., 2004; Chen et al., 2010; Wu et al., 2012). However, to date, there has been no report of SSR markers in *Hoya*. In the current study, we developed and characterized 12 transcriptome-derived SSR markers for *H. ledongensis* and tested their transferability to its congeneric species *H. jianfenglingensis*.

METHODS AND RESULTS

Fifteen individuals were sampled from each of two natural populations of *H. ledongensis* in Bawangling (CJL: 19°08′13.6″N, 109°10′37.7″E) and Baisha (BSL: 18°45′50.3″N, 108°57′48.8″E), and six individuals were sampled from the population in Jiangfengling (LDL: 18°46′27.3″N, 108°52′29.6″E) in Hainan Province. An additional six individuals of the congeneric species *H. jianfenglingensis* were collected from Bawangling (CJJ: 19°13′12.0″N, 109°08′07.6″E), Hainan Province (Appendix 1). Genomic DNA was extracted from silica-dried leaves using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). The leaf transcriptome of *H. ledongensis* was sequenced as described by Liu (2012); based on these data, we selected 46 pairs of transcriptome-derived primers with five or more repeats of dinucleotide or trinucleotide motifs.

To screen the expressed sequence tag (EST)–SSR primers, PCR amplification was conducted using two individuals from each of the two *H. ledongensis* populations (CJL and BSL) in a final reaction volume of 20 μL containing: 1.2 μL of DNA (15 ng/μL), 10 μL of 10× PCR KOD buffer, 4 μL of dNTPs (2.5 mM, Mg^{2+}), 0.6 μL (10 μM) of each primer, and 0.6 μL KOD polymerase (TOYOBO

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Table 1. Characteristics of 28 transcriptome-derived SSR loci of *Hoya ledongensis*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.	BLAST top hit description [organism]	BLAST top hit accession no.	*E*-value
SSR-4	F: GGGTGAGTCTTTGAGTGCCT R: CATGGTCCACACTAATCCCC	$(AG)_8$	151–169	54	KT206213	serovar 6b str. SLCC5334 complete genome [*Listeria welshimeri*]	AM_263198	1.900
SSR-6	F: TCTTCGTCCTGTCCTGTGTG R: CAGGTGCATGTGTAAAGGGA	$(TC)_8$	264–274	57	KT206214	protein EPIDERMAL PATTERNING FACTOR 2-like [*Nicotiana tomentosiformis*]	XM_009616771	5e-56
SSR-8	F: TGAACTCCAAGTCCAGGAGC R: TTGACGCTGAGGATGACAAG	$(AG)_8$	150–160	53	KT206215	zinc finger CCCH domain-containing protein 31 [*Sesamum*]	XR_847888	6e-10
SSR-41	F: TCTCTTCACTCCGCACACAC R: CCCATTTGACGACAACATCA	$(AT)_6$	266–284	59	KT206216	complete genome, chromosome chr1 [*Macaca fascicularis*]	LT160000	0.014
SSR-43	F: TATCGGGAGTTTCGTCCAAG R: TTTGGGAAATGGGATGATGT	$(ATC)_5$	255–268	55	KT206217	uncharacterized LOC104234001 (LOC104234001), transcript variant X5, mRNA [*Nicotiana sylvestris*]	XM_009787489	5e-117
SSR-45	F: TGTCTGATTCTCCCCTGGTC R: GCTGTTCCTCCTCCTGACAC	$(AT)_6$	269–279	56	KT206218	uncharacterized LOC107015919 (LOC107015919), mRNA [*Solanum pennellii*]	XM_015216363	3e-56
SSR-44	F: AGGCTCCCCGAGATCATTAC R: CAATTTGCTTGCTCCACAAC	$(TC)_8$	262–282	56	KT206219	exocyst complex component EXO70B1 (LOC102586317), transcript variant X2, mRNA [*Solanum tuberosum*]	XM_006338307	0.000
SSR-46	F: CTGCCCTGTCAAGAAGAAGG R: GACTGAACCCAGTGCTGTCA	$(TCA)_6$	239–314	56	KT206220	probable WRKY transcription factor 40 (LOC105165167), mRNA [*Sesamum indicum*]	XM_011084080	7e-82
SSR-31	F: CCCTTGCCAAATTTCTGTGT R: AAAACCCCCTTGCTGATCTT	$(TA)_6$	241–257	56	KT206221	lysosomal beta glucosidase-like (LOC105168898), mRNA [*Sesamum indicum*]	XM_011089093	0.000
SSR-17	F: TGTGAGGTGAAAAGGGTGAA R: TCCCACCAGTCAAATGTTGT	$(AT)_8$	118–126	57	KT206223	vinckei hypothetical protein partial mRNA [*Plasmodium vinckei*]	XM_008627391	2.700
SSR-14	F: CCCTGAAAATAGGCAGGAAA R: TATAGGGTTCACACGCAATG	$(AT)_8$	142–160	56	KT206224	DNA chromosome 4, contig fragment No. 14 [*Arabidopsis thaliana*]	AL161502	4e-05
SSR-23	F: CAACAATGTAGCTAACTTTGAGGTC R: CATCATGCTCACCAAGTGCT	$(TA)_8$	124–154	56	KT206222	contig VV78X055473.3, whole genome shotgun sequence [*Vitis vinifera*]	AM483450	5.0
SSR-2	F: GGTGGTCTTAGGGATGGCTAC R: TCCCTTTCTCACATCCCTGT	$(AT)_8$	240	53	KX084524	genome assembly *P. xenopodis* South Africa, scaffold PXEA contig0165619 [*Protopolystoma xenopodis*]	LM904264	0.13
SSR-12	F: TCAAGTTTTCATGGCTGCTG R: GGCAATCAAGGGGAGGTAAT	$(AG)_8$	236	53	KX084525	contig VV78X093246.6, whole genome shotgun sequence [*Vitis vinifera*]	AM462769	5e-06
SSR-13	F: AGGACACTGTTCCACCACAG R: CTCTATCTTCGTAGGCCCCC	$(GA)_8$	149	59	KX084526	genomic chromosome, chr_6 [*Cucumis melo*]	LN713260	5e-04
SSR-15	F: TTTCCACAAATTCCCCTTTTT R: AATGGAGAGGAAGCAAGCTG	$(TG)_7$	151	56	KX084527	chromosome ch10 [*Solanum lycopersicum*]	HG975522	0.17
SSR-16	F: CACACACTTGCACTTGTAGCTATG R: TAACACAAATCACCCACCCA	$(TA)_8$	154	54	KX084528	genomic scaffold, anchoredscaffold0000 [*Cucumis melo*]	LN681932	0.022
SSR-20	F: ATCAAAATTCGGAGGCTTCA R: AAGTCCCCTACTTTCTCCGC	$(TA)_8$	247	56	KX084529	clone BAC 043A03 complete sequence [*Saccharum* hybrid cultivar R570]	KF184749	0.083
SSR-25	F: CTGTCCAGCTAAACCCCCTT R: CCCAGCCAAAGAAAAGACAA	$(AG)_8$	212	56	KX084530	genome assembly T_regenti_v1_0_4, scaffold TRE_scaffold0011240 [*Trichobilharzia regenti*]	LL011240	2e-04
SSR-27	F: TGGGAAGGTGCATTTTCAG R: TCACCAGCTGTGGCAAATAC	$(GA)_8$	354	56	KX084531	chromosome 2 [*Arabidopsis thaliana*]	CP002685	4.4
SSR-28	F: CTCCACCCAAGCAAGTCCTA R: CCATAGAAAATGCCAACGGT	$(CT)_9$	196	56	KX084532	cation/H(+) antiporter 24-like (LOC104228225), mRNA [*Nicotiana sylvestris*]	XM_009780654	0.008
SSR-30	F: TCCCTGTGTCTCCCCAATTA R: TGGGATTCTTCCCACCAATA	$(CT)_9$	157	53	KX084533	genome assembly S_erinaceieuropaei, scaffold SPER contig 0084196 [*Spirometra erinaceieuropaei*]	LN300345	1e-05
SSR-34	F: GAAATGCAGGGAATGATTGAA R: GACACCGGGACCAATCTTTA	$(AG)_8$	276	54	KX084534	protein DCL, chloroplastic-like (LOC105168792), mRNA [*Sesamum indicum*]	XM_011088943	2e-49
SSR-35	F: AAGGCCAATTCTTGTTGGTG R: TCCATGCATTTGATTTTCCA	$(TA)_7$	275	54	KX084535	6-phosphogluconate dehydrogenase, decarboxylating 3, transcript variant X3, mRNA [*Nicotiana sylvestris*]	XM_009776029	0.0
SSR-36	F: TCACCATGTCCATGGAAAATTA R: TTTTTGGTTCGTAGGCTGCT	$(AT)_6$	276	57	KX084536	LP174 ribosomal protein L16 (*rpl16*) gene, partial sequence; chloroplast gene for chloroplast product [*Stewartia serrata*]	AY070303	0.017
SSR-37	F: TGCTGCACTTTAACCGAAGA R: TGTTTGGTGCTGTTGAGAAGA	$(ATT)_6$	276	56	KX084537	auxin response factor 9 (LOC105165118), mRNA [*Sesamum indicum*]	XM_011084003	0.0

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Table 1. Continued.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.	BLAST top hit description [organism]	BLAST top hit accession no.	*E*-value
SSR-40	F: CGCAGAGGCATATGGAATTT R: ATACAAATGAGGCGAGGCTG	$(TA)_6$	252	54	KX084538	SPX domain-containing protein 4 (LOC105167645), mRNA [*Sesamum indicum*]	XM_011087437	3e-158
SSR-42	F: GCCCAGAACCCTCATTCATA R: GACAAGGCCCTCAGGTATCA	$(GA)_8$	263	52	KX084539	uncharacterized LOC105179451 (LOC105179451), mRNA [*Sesamum indicum*]	XM_011103071	0.0

Note: T_a = annealing temperature.

Ideas & Chemistry, Osaka, Japan). PCR was performed under standard conditions for all primers using the following cycling conditions: 3 min of denaturation at 94°C; followed by 30 cycles of 40 s at 94°C, 45 s at the annealing temperature of each primer set, and 40 s at 72°C; with a final extension of 10 min at 72°C. The PCR products were first resolved with electrophoresis in a 1.5% agarose gel to assess if the amplification was successful for the expected sized products of each primer pair. These experiments produced PCR products with expected sizes that were successfully amplified from 28 primer pairs designed for *H. ledongensis*.

To test the polymorphism level of these 28 primer pairs, PCR was conducted using all of the *H. ledongensis* and *H. jianfenglingensis* samples in a final reaction volume of 20 µL, using the conditions detailed above. Amplified products were resolved in an 8% polyacrylamide gel electrophoresis (PAGE), and gels were stained with 0.1% silver nitrate. The band size was calculated by comparison with a 50-bp DNA ladder (TaKaRa Biotechnology Co., Dalian, China). Our results showed that 12 transcriptome-derived SSR markers were polymorphic in *H. ledongensis* (Table 1).

POPGENE version 1.31 (Yeh et al., 1999) was used to calculate the population genetics parameters for *H. ledongensis* and *H. jianfenglingensis*, respectively. Two to 11 alleles were detected for these loci (Table 2). These polymorphic loci had observed heterozygosity from 0.133 to 0.867 and expected heterozygosity from 0.128 to 0.894, respectively.

Out of the 12 polymorphic microsatellite loci, six, seven, and one in the CJL, BSL, and LDL populations of *H. ledongensis*, respectively, exhibited significant deviations from Hardy–Weinberg equilibrium (HWE; Table 2). The deviations from HWE might be an effect of null alleles at these loci, despite the fact that null homozygous individuals were absent in these populations. To test this, we used MICRO-CHECKER (version 2.2.3; van Oosterhout et al., 2004) to check if there were null alleles. We found that null alleles were present at five markers (SSR-8, SSR-41, SSR-45, SSR-46, and SSR-31) in the population BSL, at three markers (SSR-8, SSR-31, and SSR-23) in the population CJL, and at one marker (SSR-14) in the population CJJ. Because HWE was also observed in populations with null alleles at some loci, homozygote excess of populations should be another factor for the deviations from HWE. No significant linkage disequilibrium was observed between these markers; therefore, they can be considered independent across the genome. Furthermore, cross-species amplification of these 12 markers was successful in *H. jianfenglingensis* and only one locus in the CJJ population exhibited a significant deviation from HWE.

CONCLUSIONS

To the best of our knowledge, this is the first study to report SSR markers in a species of *Hoya*. We have identified and verified 12 markers for *H. ledongensis* that can also be used for the investigation of its congener species *H. jianfenglingensis*. The primers designed in this study can be applied to the investigation of genetic diversity and population structure of *Hoya* and other related species. Furthermore, natural hybridization between *Hoya* species can be tested with these markers. This work provides an important tool for the development of scientific conservation strategies and testing natural hybridization hypotheses in *Hoya*.

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Table 2. Genetic diversity of 12 polymorphic markers developed in three populations of *Hoya ledongensis* and one population of *H. jianfenglingensis*.[a]

	H. ledongensis									*H. jianfenglingensis*		
	CJL (N = 15)			BSL (N = 15)			LDL (N = 6)			CJJ (N = 6)		
Locus	A	H_o	H_e[b]	A	H_o	H_e[b]	A	H_o	H_e[b]	A	H_o	H_e[b]
SSR-4	4	0.333	0.404	4	0.467	0.728	4	0.500	0.772	3	0.500	0.681
SSR-6	2	0.133	0.128	4	0.400	0.673	3	0.333	0.667	3	0.333	0.318
SSR-8	6	0.533	0.747*	3	0.466	0.662***	3	0.833	0.681*	3	0.833	0.681
SSR-41	4	0.400	0.605	4	0.200	0.659*	2	0.500	0.409	4	0.833	0.772
SSR-43	2	0.533	0.405	5	0.333	0.694	2	0.500	0.409	2	0.333	0.303
SSR-45	5	0.333	0.740	4	0.466	0.705	2	0.333	0.484	3	0.333	0.439
SSR-44	6	0.867	0.811	8	0.733	0.795***	3	0.500	0.439	2	0.333	0.303
SSR-46	5	0.600	0.678***	11	0.533	0.894***	2	0.666	0.545	3	0.666	0.727*
SSR-31	5	0.400	0.698***	3	0.266	0.625**	2	0.333	0.484	2	0.333	0.303
SSR-17	2	0.866	0.508*	4	0.400	0.600*	2	0.166	0.166	2	0.333	0.303
SSR-14	4	0.533	0.652*	4	0.400	0.512	3	0.833	0.727	5	0.333	0.742
SSR-23	4	0.333	0.627*	4	0.266	0.643***	2	0.333	0.303	5	0.333	0.742

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals analyzed.

[a] Population and voucher information are provided in Appendix 1.

[b] Significant deviations from Hardy–Weinberg equilibrium after sequential Bonferroni corrections: *** represents significance at 0.1% nominal level; ** represents significance at 1% nominal level; * represents significance at 5% nominal level.

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Appendix 1. Voucher and location information for the *Hoya* species and populations used in this study. All voucher specimens are deposited at the herbarium of South China Agricultural University (CANT), Guangzhou, China.

Species	Population code	Voucher no.	Collection locality	Geographic coordinates	N
Hoya ledongensis Shao Y. He & P. T. Li	CJL	LSYH20110924	Changjiang, Hainan, China	19°08′13.6″N, 109°10′37.7″E	15
	BSL	LSYH20110928	Ledong, Hainan, China	18°45′50.3″N, 108°57′48.8″E	15
	LDL	LSYH20110926	Changjiang, Hainan, China	18°46′27.3″N, 108°52′29.6″E	6
Hoya jiangfenglingensis Shao Y. He & P. T. Li	CJJ	JSYH20110924	Changjiang, Hainan, China	19°13′12.0″N, 109°08′07.6″E	6

Note: N = number of individuals sampled.

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