**PRIMER NOTE**

**DEVELOPMENT OF TRANSCRIPTOME-DERIVED SSR MARKERS FOR *HOYA LEDONGENSIS* (**APOCYNACEAE**) AND CROSS-AMPLIFICATION IN A CONGER**

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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* (**Apo**cynaceae), 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. (**Apo**cynaceae) 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, 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 (CJL: 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, Mg²⁺), 0.6 μL (10 μM) of each primer, and 0.6 μL KOD polymerase (TOYOBO).
Characteristics of 28 transcriptome-derived SSR loci of *Hoya ledongensis*

| Locus | Repeat size (bp) | Tm (°C) | Allele size range (bp) | BLAST top hit accession no. | GenBank accession no. | BLAST top hit description (organism) |
|-------|-----------------|---------|-----------------------|-----------------------------|----------------------|--------------------------------------|
| SR-4  | (AG)₈            | 151–169 | 152–169               | AM_263198                   | KT006313             | Listeria welshimeri complete genome |
| SR-6  | (AG)₈            | 255–288 | 259–282               | KT006317                    | KT006315             | Listeria welshimeri complete genome |
| SR-8  | (AG)₈            | 254–288 | 258–282               | KT006318                    | KT006316             | Listeria welshimeri complete genome |
| SR-11 | (AG)₈            | 198–224 | 201–223               | KT006320                    | KT006319             | Listeria welshimeri complete genome |
| SR-14 | (AG)₈            | 250–280 | 254–280               | KT006321                    | KT006322             | Listeria welshimeri complete genome |
| SR-17 | (AG)₈            | 312–342 | 316–342               | KT006323                    | KT006324             | Listeria welshimeri complete genome |
| SR-20 | (AG)₈            | 292–322 | 296–322               | KT006325                    | KT006326             | Listeria welshimeri complete genome |
| SR-23 | (AG)₈            | 250–280 | 254–280               | KT006327                    | KT006328             | Listeria welshimeri complete genome |
| SR-26 | (AG)₈            | 250–280 | 254–280               | KT006329                    | KT006330             | Listeria welshimeri complete genome |
| SR-29 | (AG)₈            | 250–280 | 254–280               | KT006331                    | KT006332             | Listeria welshimeri complete genome |
| SR-32 | (AG)₈            | 250–280 | 254–280               | KT006333                    | KT006334             | Listeria welshimeri complete genome |
| SR-35 | (AG)₈            | 250–280 | 254–280               | KT006335                    | KT006336             | Listeria welshimeri complete genome |
| SR-38 | (AG)₈            | 250–280 | 254–280               | KT006337                    | KT006338             | Listeria welshimeri complete genome |
| SR-41 | (AG)₈            | 250–280 | 254–280               | KT006339                    | KT006340             | Listeria welshimeri complete genome |
| SR-44 | (AG)₈            | 250–280 | 254–280               | KT006341                    | KT006342             | Listeria welshimeri complete genome |
| SR-47 | (AG)₈            | 250–280 | 254–280               | KT006343                    | KT006344             | Listeria welshimeri complete genome |

http://www.bioone.org/loi/apps
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 CJJ, 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*.

### LITERATURE CITED

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| Locus | SSR-40 | SSR-42 |
|------|-------|-------|
| E-value | 3e-158 | 0.0 |
| BLAST top hit accession no. | XM_011087437 | XM_011103071 |
| GenBank BLAST top hit description (organism) | Sesamum indicum | Sesamum indicum |
| GenBank accession no. | XM_011087437 | XM_011103071 |
| Description (organism) | Sesamum indicum | Sesamum indicum |
| Repeat motif | (GA) 8 | uncharacterized LOC105179451 (LOC105179451), mRNA |
| Allele size (bp) | 252 | 263 |
| Note: $T_a$ = annealing temperature | | |
Table 2. Genetic diversity of 12 polymorphic markers developed in three populations of *Hoya ledongensis* and one population of *H. jianfenglingensis.\(^a\)

| Locus | CJL (N = 15) | BSL (N = 15) | LDL (N = 6) | H. jianfenglingensis (N = 6) |
|-------|--------------|--------------|--------------|-----------------------------|
|       | A | \(H_o\) | \(H_e\) | A | \(H_o\) | \(H_e\) | A | \(H_o\) | \(H_e\) | A | \(H_o\) | \(H_e\) |
| SSF-4  | 4 | 0.333 | 0.404 | 4 | 0.400 | 0.673 | 4 | 0.500 | 0.772 | 3 | 0.500 | 0.681 |
| SSF-6  | 2 | 0.133 | 0.128 | 4 | 0.400 | 0.673 | 3 | 0.333 | 0.667 | 3 | 0.333 | 0.318 |
| SSF-8  | 6 | 0.533 | 0.747* | 3 | 0.466 | 0.662*** | 3 | 0.833 | 0.681* | 4 | 0.833 | 0.772 |
| SSF-11 | 5 | 0.333 | 0.740 | 4 | 0.466 | 0.705 | 2 | 0.333 | 0.484 | 3 | 0.333 | 0.303 |
| SSF-17 | 6 | 0.867 | 0.508* | 5 | 0.333 | 0.694 | 2 | 0.666 | 0.545 | 2 | 0.333 | 0.303 |
| SSF-23 | 4 | 0.333 | 0.627* | 4 | 0.466 | 0.643*** | 2 | 0.333 | 0.303 | 5 | 0.333 | 0.742 |

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

\(^a\)Population and voucher information are provided in Appendix 1.

**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* | CJL             | LSZH20110924 | Changjiang, Hainan, China | 19°08’13.6"N, 109°10’37.7"E | 15  |
|                    | BSL             | LSZH20110928 | Ledong, Hainan, China | 18°45’50.3"N, 108°57’48.8"E | 15  |
|                    | LDL             | LSZH20110926 | Changjiang, Hainan, China | 18°46’27.3"N, 108°52’29.6"E | 6   |
| *Hoya jianfenglingensis* | CJL             | JSYH20110924 | Changjiang, Hainan, China | 19°13’12.0"N, 109°08’07.6"E | 6   |

**Note:** N = number of individuals sampled.