Applications in Plant Sciences

Botanical Society of America
leading scientists and educators since 1893

PRIMER NOTE

Development and characterization of polymorphic EST-SSR markers for *Paphiopedilum henryanum* (Orchidaceae)

Yufeng Xu[1], Ruidong Jia[1,2], Yanhui Zhou[1], Hao Cheng[1], Xin Zhao[1], and Hong Ge[1,2]

Manuscript received 21 November 2017; revision accepted 2 February 2018.

[1] Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences/Key Laboratory of Horticultural Crop Biology and Germplasm Creation of the Ministry of Agriculture, Beijing 100081, People's Republic of China

[2] Authors for correspondence: jiaruidong@caas.cn, gehong@caas.cn

Citation: Xu, Y., R. Jia, Y. Zhou, H. Cheng, X. Zhao, and H. Ge. 2018. Development and characterization of polymorphic EST-SSR markers for *Paphiopedilum henryanum* (Orchidaceae). *Applications in Plant Sciences* 6(5): e1152.

doi:10.1002/aps3.1152

PREMISE OF THE STUDY: Microsatellite primers were developed for *Paphiopedilum henryanum* (Orchidaceae), a species threatened with extinction, to assess genetic diversity and population genetic structure.

METHODS AND RESULTS: Based on the transcriptome data of *P. henryanum*, 34 novel polymorphic microsatellite expressed sequence tag–simple sequence repeat markers were developed and characterized in 33 individuals from two *P. henryanum* populations. The results showed the number of alleles per locus ranged from two to four, and levels of observed and expected heterozygosity per locus ranged from 0.000 to 1.000 and from 0.000 to 0.7333, respectively. Of these markers, some primers showed good amplification results in seven other *Paphiopedilum* species.

CONCLUSIONS: The developed microsatellite markers will be useful in exploring the genetic diversity and structure of *P. henryanum*. Furthermore, most loci were successfully cross-amplified in seven other species of *Paphiopedilum*, indicating that they will be of great value for genetic study across this genus.

KEY WORDS microsatellite markers; Orchidaceae; *Paphiopedilum henryanum*; polymorphism; transcriptome.

Orchidaceae is a diverse family of flowering plants that is widely known among botanists and horticulturalists for its often showy, fragrant flowers with varied morphologies. One genus within Orchidaceae, *Paphiopedilum* Pfitzer (subfamily Cypripedioideae), are characterized as lady's slipper orchids because of their floral morphology, with 96 accepted species (Cox et al., 1997; Guo et al., 2015). *Paphiopedilum* has attracted attention from hobbyists and biologists because of its specialized floral traits and the mode of pollination by deceit. Recently, populations of *Paphiopedilum* have faced a rapid decline, becoming endangered as a result of its narrow distribution, climate change, habitat loss, and overcollection for their beautiful, unique flowers (Zhang et al., 2016). All species within this genus are listed in Appendix I of the Convention on International Trade in Endangered Species (CITES, https://cites.org/; Sun et al., 2011), thus these species cannot be traded internationally for primarily commercial purposes (Zeng et al., 2012). *Paphiopedilum henryanum* Braem is distributed in crevices of shady cliffs or rocks and well-drained habitats in the mountains along the Sino-Vietnamese border. The natural range of *P. henryanum*, a popular ornamental, has declined significantly as a result of habitat destruction and excessive collection. Therefore, genetic information, such as genetic diversity and population structure, is important for the conservation of this species.

Microsatellites or simple sequence repeat (SSR) markers can be identified using genomes or transcriptomes; they have become one of the most important genetic markers in plant genetic analyses because of their hypervariability, multiallelic nature, codominant inheritance, and reproducibility (Powell et al., 1996). Expressed sequence tag (EST)–SSRs are a part of genes, and may help to predict candidate functional genes (Varshney et al., 2005). Li et al. (2010) isolated 10 polymorphic microsatellite loci from genomes in *P. concolor* (Bateman) Pfitzer (*Paphiopedilum* subgen. *Brachypetalum*). We verified that only two markers can be successfully amplified in *P. henryanum* (subgen. *Paphiopedilum*). This poor amplification could be the result of the large genetic distance between *P. henryanum* and *P. concolor*, as the species are not in the same subgenus. Moreover, microsatellite markers have not yet been developed from transcriptomes for *Paphiopedilum* or for the identification and assessment of the genetic diversity of *P. henryanum*. In this study, we developed 34 novel microsatellite markers for *P. henryanum*. These 34 polymorphic markers were tested on 33 individuals from two populations of *P. henryanum*, and their transferability was tested in seven other *Paphiopedilum* species, which were selected according to the infrageneric classification (Lang et al., 2006) and the DNA barcoding of *Paphiopedilum* (Guo et al., 2016).

TABLE 1. Characteristics of 34 polymorphic microsatellite loci developed for *Paphiopedilum henryanum*.

Locus	Primer sequences (5′–3′)	T_a (°C)	Repeat motif	Allele size range (bp)	GenBank accession no.	Putative function [organism] (BLAST)	*E*-value
PH_SSR011	F: CGAAGCACGGGTCTCTTTCT R: ACCACCGACATTACCTGCAG	60	$(TTC)_7$	107	MG333695	—	—
PH_SSR025	F: AATTTCTTCCAGGAGGGCGG R: TCGGCTGTCCATGCTTGTAG	60	$(GACA)_5$	101	MG333696	—	—
PH_SSR026	F: TCATCCCTTCATCCCGAGGT R: TCCCCATGCTCGAAGCTTTT	60	$(ACG)_6$	248	MG333697	PREDICTED: uncharacterized LOC110115938 [*Dendrobium catenatum*]	2E-37
PH_SSR041	F: CTGCTAGCCTGAGGAGAACG R: CAAGAAACCCCCACCTCCTC	60	$(CTC)_7$	101	MG333698	PREDICTED: uncharacterized LOC110098091 [*Dendrobium catenatum*]	0
PH_SSR060	F: TACCTCCATCACTCTCGCCT R: TTGCTCGCCTTTTTGACAGC	60	$(AC)_{10}$	127	MG333699	—	—
PH_SSR172	F: GGCCAAGTACATGCACCCAT R: TTCCCACCTCGGTTATGCAC	60	$(GCC)_6$	278	MG333700	PREDICTED: nuclear transcription factor Y subunit B-1-like LOC110037676 [*Phalaenopsis equestris*]	8E-95
PH_SSR272	F: GCAAATCCATTCAGCCCTGC R: CGACATGGTCTGAGAGGAGC	60	$(CAG)_6$	193	MG333701	PREDICTED: probable WRKY transcription factor 21 LOC110107315 [*Dendrobium catenatum*]	0
PH_SSR334	F: CACTGGGGGATCTTCGAAGG R: CAGCACCTCTTCGCGTAAGA	60	$(GCG)_7$	185	MG333702	PREDICTED: protein LURP-one-related 8-like LOC110034281 [*Phalaenopsis equestris*]	1E-56
PH_SSR343	F: CTTGGGACTCTTTCCTCGGC R: CCAGGAGGCTCTCAGCTTTC	60	$(AGA)_6$	250	MG333703	PREDICTED: uncharacterized LOC110096658 [*Dendrobium catenatum*]	3E-71
PH_SSR351	F: CTCCCAATCTCCACCGACAA R: TGGATCCCAGGGAAGGTAGG	60	$(CGG)_6$	259	MG333704	PREDICTED: scarecrow-like protein 15 LOC110024574 [*Phalaenopsis equestris*]	3E-33
PH_SSR356	F: TGCAGATGAGCCCATGCATT R: TCACGCCTGTATTTCTGCGT	60	$(GAA)_6$	260	MG333705	PREDICTED: uncharacterized LOC110100801 [*Dendrobium catenatum*]	0
PH_SSR372	F: TTGATTGGCGCAGTGAAAGC R: CCACTGATCGCAGACCATCA	60	$(GGC)_6$	266	MG333706	PREDICTED: uncharacterized LOC110101380 [*Dendrobium catenatum*]	5E-141
PH_SSR376	F: GGCACTTACAGCAAGGCTCT R: GAGACCTGGGCCCATCAAAA	60	$(CTG)_6$	102	MG333707	PREDICTED: uncharacterized LOC110109757 [*Dendrobium catenatum*]	4E-80
PH_SSR383	F: ACGCGGCAAAAATGATGAGG R: AGGAGGGTTCATGCAGTAGT	59	$(TCA)_6$	100	MG333708	PREDICTED: ras GTPase-activating protein-binding protein 1-like LOC110103447 [*Dendrobium catenatum*]	7E-40
PH_SSR413	F: CAGGCTCCAAAACAAGGCAC R: GGGACTGGGGAGTAAAAGGC	60	$(CAG)_7$	263	MG333709	PREDICTED: uncharacterized LOC110096393 [*Dendrobium catenatum*]	1E-98
PH_SSR416	F: CAGGTTGACAGCAATGTCGC R: GCCGCAGCTTTTCGGATAAG	60	$(CCG)_6$	188	MG333710	PREDICTED: G-type lectin S-receptor-like serine/threonine-protein kinase SD2-2 LOC110111128 [*Dendrobium catenatum*]	0
PH_SSR425	F: AGTAGAGGATCTGGTCAACGGA R: TGCAAGGTTCTAGAGTGCATGA	60	$(CAT)_7$	240	MG333711	—	—
PH_SSR426	F: AGCGTGTTTGGACTAGAGCA R: TCGGGGATGCACATGGAAAA	60	$(AAAC)_5$	239	MG333712	—	—
PH_SSR430	F: GCTCCATAGCTGGCGATCAT R: TCTTTCTGACGGCGCAAGAT	60	$(GA)_9$	157	MG333713	PREDICTED: phosphoribosylformylglycinamidine cyclo-ligase, chloroplastic/mitochondrial LOC110097203 [*Dendrobium catenatum*]	0
PH_SSR447	F: GGGTGGGGAGAGTAGGAGTT R: GCCACAACTTGTTTTCCCGG	60	$(CGG)_6$	219	MG333714	PREDICTED: probable serine/threonine-protein kinase PBL21 LOC110111545 [*Dendrobium catenatum*]	7E-128

(Continues)

TABLE 1. (Continued)

Locus	Primer sequences (5′–3′)	T_a (°C)	Repeat motif	Allele size range (bp)	GenBank accession no.	Putative function [organism] (BLAST)	*E*-value
PH_SSR535	F: TTCGTCCTCACTTCTTCGCC R: GAAGAGGAGATGGCTCGTGG	60	$(CCT)_6$	157	MG333715	PREDICTED: uncharacterized LOC110108101 [*Dendrobium catenatum*]	3E-177
PH_SSR547	F: CCACGTGTGACAGAATCCCA R: GGCTCCCGACGAGGAATTAC	60	$(CGT)_6$	235	MG333716	PREDICTED: probable aspartyl protease At4g16563 LOC110106324 [*Dendrobium catenatum*]	0
PH_SSR548	F: TCAACAAGGACGGGTTGGTC R: TTGATGTGGCACAAGCACAC	60	$(ATC)_6$	176	MG333717	—	—
PH_SSR592	F: AGAGAAGGAGCACCAATGGC R: CTTCGATGGATCATGGGCGA	60	$(GCT)_6$	230	MG333718	PREDICTED: scarecrow-like protein 27 LOC110031666 [*Phalaenopsis equestris*]	2E-76
PH_SSR645	F: CCACCAGCTTTCATATCCTCCA R: GCCCATGCTGTGCAAAAAGA	60	$(ATC)_6$	231	MG333719	PREDICTED: uncharacterized LOC110103522 [*Dendrobium catenatum*]	9E-06
PH_SSR651	F: AAGAAGTGGCTTCCATGGCA R: GCAAAACCAAGGTGTCGTCC	60	$(TCT)_6$	215	MG333720	PREDICTED: NAC domain-containing protein 92-like LOC110029752 [*Phalaenopsis equestris*]	4E-59
PH_SSR658	F: AGCCAACACAGCCACGATAA R: AAGAACCATCACCACCACCC	60	$(GCT)_7$	190	MG333721	—	—
PH_SSR669	F: CAAACCTCGCTCGGAAGACT R: AGGGTTTCTATCGCTTGGCC	60	$(TCGAC)_6$	259	MG333722	PREDICTED: RNA polymerase II C-terminal domain phosphatase-like 3 LOC110024905 [*Phalaenopsis equestris*]	0
PH_SSR687	F: GCTGCCAATTCGAATGGAGG R: GCTGCCGATTCTCCTTCCTT	60	$(GGC)_6$	203	MG333723	—	—
PH_SSR698	F: GAAAACCGATTGGCGTCGAG R: TTCCCTTCTCCCATTTCCGG	60	$(GA)_9$	192	MG333724	PREDICTED: protein SCO1 homolog 1, mitochondrial LOC110110179 [*Dendrobium catenatum*]	0
PH_SSR716	F: AGCTATGAGGAACTGCGCTG R: TGTTGACATGATCCGTGGCA	60	$(GGC)_6$	182	MG333725	PREDICTED: calcium uniporter protein 2, mitochondrial-like LOC110093017 [*Dendrobium catenatum*]	2E-118
PH_SSR800	F: AGCTTGAAGTACTTGGGGGC R: TCCACCTTCTCCTCCTCACT	60	$(TGG)_8$	146	MG333726	PREDICTED: uncharacterized LOC110021639 [*Phalaenopsis equestris*]	0
PH_SSR809	F: CCTTCCTCATGATCCGTCCG R: TTAGCTCTCTCCCCTCCGAG	60	$(CGC)_6$	159	MG333727	PREDICTED: nuclear transcription factor Y subunit B-1-like LOC110036525 [*Phalaenopsis equestris*]	4E-70
PH_SSR877	F: GGCGATTGACCTCTGCTGAT R: TCGGCTTTGGTTGCTGAGAT	60	$(CGC)_6$	217	MG333728	PREDICTED: probable WRKY transcription factor 27 LOC110105927 [*Dendrobium catenatum*]	8E-96

Note: — = not found (*E*-value >10^{-6}); T_a = annealing temperature.

METHODS AND RESULTS

Floral organs of *P. henryanum* were gathered from the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (Appendix 1), immediately frozen in liquid nitrogen, and then stored at −80°C. Complete floral organs were sent to the Novogene Technology Co. (Beijing, China) to extract RNA and construct a cDNA library. Transcriptome sequencing was conducted using an Illumina HiSeq 2000 (Illumina, San Diego, California, USA). The raw data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRP131426, PRJNA431671). Then, the MIcroSAtellite identification tool (MISA; Thiel et al., 2003) was used to screen for SSR motifs from all unigenes, and the minimum numbers of repeats were set as seven, five, five, five, and five for di-, tri-, tetra-, penta-, and hexanucleotide repeats, respectively. MISA recovered a total of 25,255 SSR motifs, with mononucleotide repeats (15,543, 61.54%) being the most common, followed by dinucleotide (6238, 24.70%), trinucleotide (2313, 9.16%), complex repetitive type (1039, 4.11%), tetranucleotide (97, 0.38%), hexanucleotide (16, 0.06%), and pentanucleotide (9, 0.04%) repeats. Primer3 software (Untergasser et al., 2012) was used to design primer pairs with lengths of 17–24 bases, PCR product size ranging from 100–350 bp, and annealing temperatures of 55–62°C; 906 primer pairs were designed. We then initially screened 129 pairs using two *P. henryanum* individuals (Appendix 1).

Paphiopedilum henryanum is distributed in Malipo and Maguan counties in Yunnan Province, China (Lang et al., 2006). During our field investigation, we found only two wild populations in Malipo County: Danong (DN) and Taiyangchong (TYC). *Paphiopedilum henryanum* has become locally extinct in locations recorded in previous literature, including Dulong and Shanche in Maguan County, Tusicheng in Wenshan County, and Liangzicun in Xichou County. Harvesting leaves can minimize damage to plants, thus we collected leaf tissue from 25 individuals from the DN population and eight individuals from the TYC population (Appendix 1). Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Allen et al., 2006). PCR amplifications were performed in 20-μL reaction mixtures containing 0.5 units of *Taq* polymerase (TaKaRa Biotechnology Co., Dalian, China), 2 μL of 10× PCR buffer (200 mM Tris-HCl [pH 8.8], 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 1% Triton X-100, 20 mM $MgSO_4$), 1.6 μL of dNTPs (2.5 mM each), 0.5 μL of each primer (10 μM), and 1 μL of genomic DNA (~30–50 ng/μL). PCR conditions comprised an initial denaturing step at 94°C for 5 min; followed by 10 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 40 s; 15 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 40 s; 10 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 40 s; and a final extension at 72°C for 7 min. PCR products were electrophoresed on 8% polyacrylamide denaturing gel and visualized using silver staining. Polymorphisms of these 129 loci were evaluated using the 33 *P. henryanum* individuals from the DN and TYC populations; we obtained 34 polymorphic EST-SSR markers (Table 1), and the other 95 loci did not amplify well. The efficiency of the 34 polymorphic markers in cross-species amplification was tested.

The 34 polymorphic SSR markers were analyzed using the software POPGENE 32 (version 1.32; Yeh, 1997) to calculate the average number of alleles, observed heterozygosity, expected heterozygosity, and likelihood ratio test for Hardy–Weinberg equilibrium. The number of alleles per locus ranged from two to four alleles, with a mean of 2.32 alleles per locus. The levels of observed and expected heterozygosity ranged from 0.000 to 1.000 and from 0.000 to 0.7333, with averages of 0.3808 and 0.3636, respectively. Nine loci in the DN population and six loci in the TYC population showed significant deviation from Hardy–Weinberg equilibrium (Table 2). Cross-species amplification of the 34 polymorphic markers was tested in seven related species (five individuals for each population; Appendix 1), and some markers were successfully cross-amplified in *P. villosum* (Lindl.) Stein (29, 85.29%), *P. hirsutissimum* (Lindl. ex Hook. f.) Stein (28; 82.35%), *P. venustum* (Sims) Pfitzer (28, 82.35%), *P. wardii* Summerh. (25, 73.53%), *P. dianthum* Tang & F. T. Wang (23, 67.65%), *P. concolor* (19, 55.88%), and *P. micranthum* Tang & F. T. Wang (19, 55.88%; Table 3).

CONCLUSIONS

The 34 microsatellite markers described here are the first developed from the *P. henryanum* transcriptome. These newly described

TABLE 2. Polymorphism of the 34 EST-SSRs in two populations of *Paphiopedilum henryanum*.[a]

	Danong population ($N = 25$)			Taiyangchong population ($N = 8$)		
Locus	A	H_o	H_e[b]	A	H_o	H_e[b]
PA_SSR011	2	0.160	0.150	2	0.250	0.500
PA_SSR025	1	0.000	0.000	3	0.500	0.433
PA_SSR026	3	0.160	0.222*	2	0.125	0.458*
PA_SSR041	3	0.560	0.448	2	0.125	0.125
PA_SSR060	3	1.000	0.637	3	1.000	0.633*
PA_SSR172	2	0.160	0.327*	3	0.375	0.425
PA_SSR272	2	0.480	0.372	3	0.250	0.242
PA_SSR334	3	0.174	0.165	2	0.500	0.400
PA_SSR343	2	0.640	0.509	2	0.375	0.325
PA_SSR351	3	0.160	0.571**	1	0.000	0.000
PA_SSR356	2	0.280	0.246	2	0.250	0.233
PA_SSR372	1	0.000	0.000	2	0.125	0.525*
PA_SSR376	2	0.800	0.490**	2	0.125	0.525*
PA_SSR383	3	0.625	0.592	3	0.625	0.508
PA_SSR413	2	0.040	0.040	2	0.625	0.458
PA_SSR416	2	0.320	0.274	2	0.500	0.400
PA_SSR425	2	0.440	0.393	3	0.125	0.342
PA_SSR426	2	0.400	0.327	2	0.250	0.233
PA_SSR430	2	0.640	0.444**	2	0.500	0.400
PA_SSR447	2	0.080	0.078	1	0.000	0.000
PA_SSR535	3	0.280	0.365	3	1.000	0.675
PA_SSR547	2	0.240	0.216	2	0.500	0.400
PA_SSR548	2	0.440	0.350	3	0.750	0.575
PA_SSR592	2	0.120	0.115	2	0.125	0.125
PA_SSR645	1	0.000	0.000	2	0.500	0.400
PA_SSR651	3	0.640	0.456	3	0.500	0.425
PA_SSR658	2	0.160	0.150	4	0.750	0.733*
PA_SSR669	2	0.200	0.184	2	0.125	0.125
PA_SSR687	2	0.360	0.301	2	0.375	0.325
PA_SSR698	3	0.958	0.627**	3	0.625	0.542
PA_SSR716	3	0.520	0.438	1	0.000	0.000
PA_SSR800	4	0.640	0.693**	4	0.375	0.592
PA_SSR809	3	0.320	0.610**	3	0.500	0.625*
PA_SSR877	3	0.800	0.505**	3	0.500	0.508

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

[a]Voucher and locality information are provided in Appendix 1.

[b]Likelihood ratio test for Hardy–Weinberg equilibrium: *$P < 0.05$, **$P < 0.01$.

TABLE 3. Cross-amplification of the 34 polymorphic EST-SSR markers developed for *Paphiopedilum henryanum* in seven other species of *Paphiopedilum*.[a]

Locus	*P. villosum*	*P. hirsutissimum*	*P. venustum*	*P. wardii*	*P. dianthum*	*P. concolor*	*P. micranthum*
PH_SSR011	+	+	+	+	+	+	—
PH_SSR025	+	—	+	—	—	—	—
PH_SSR026	—	+	+	+	—	—	—
PH_SSR041	+	+	+	—	—	—	+
PH_SSR060	+	—	+	—	—	—	—
PH_SSR172	+	—	+	—	+	+	—
PH_SSR272	—	+	+	+	+	+	+
PH_SSR334	+	+	+	+	+	+	—
PH_SSR343	+	+	—	+	+	+	—
PH_SSR351	+	—	+	+	—	—	+
PH_SSR356	—	+	+	+	—	+	+
PH_SSR372	+	+	+	+	+	—	+
PH_SSR376	+	+	+	+	+	—	—
PH_SSR383	+	+	+	+	—	+	+
PH_SSR413	+	+	+	+	—	—	+
PH_SSR416	+	+	+	+	+	+	—
PH_SSR425	+	+	—	—	—	—	+
PH_SSR426	+	+	—	+	—	—	+
PH_SSR430	+	—	+	+	+	+	—
PH_SSR447	+	+	+	+	+	—	—
PH_SSR535	+	+	+	+	+	+	+
PH_SSR547	+	+	+	—	+	+	+
PH_SSR548	+	+	+	—	+	+	—
PH_SSR592	+	+	+	+	+	+	+
PH_SSR645	+	+	+	+	+	—	+
PH_SSR651	+	+	+	+	+	+	+
PH_SSR658	+	+	+	+	+	+	—
PH_SSR669	—	+	—	+	+	—	—
PH_SSR687	+	+	—	+	—	—	+
PH_SSR698	+	—	—	—	+	+	—
PH_SSR716	+	+	+	+	+	+	+
PH_SSR800	—	+	+	—	+	+	+
PH_SSR809	+	+	+	+	+	—	+
PH_SSR877	+	+	+	+	+	+	+

Note: + = successful amplification in all individuals; — = unsuccessful amplification.
[a]Locality and voucher information are provided in Appendix 1.

markers are likely to be useful for evaluating the genetic diversity and population structure, as well as for facilitating the development of a conservation strategy for *P. henryanum*, a species that is increasingly threatened by habitat destruction. In addition, the cross-amplification of these microsatellite loci in seven other species suggests that these 34 markers have good transferability among other *Paphiopedilum* species, especially in *Paphiopedilum* subgen. *Paphiopedilum*. Therefore, the effective amplification ratio of EST-SSR markers increases with decreased genetic distance.

ACKNOWLEDGMENTS

This study is supported by the National Natural Science Foundation of China (NSFC; no. 31301810) and the Special Fund for Agroscientific Research in the Public Interest (201203071).

LITERATURE CITED

Allen, G. C., M. A. Flores-Vergara, S. Krasnyanski, S. Kumar, and W. F. Thompson. 2006. A modified protocol for rapid DNA isolation from plant tissues using cetyltrimethylammonium bromide. *Nature Protocols* 1: 2320–2325.

Cox, A. V., A. M. Pridgeon, V. A. Albert, and M. W. Chase. 1997. Phylogenetics of the slipper orchids (Cypripedioideae, Orchidaceae): Nuclear rDNA ITS sequences. *Plant Systematics and Evolution* 208: 197–223.

Guo, Y. Y., Y. B. Luo, Z. J. Liu, and X. Q. Wang. 2015. Reticulate evolution and sea-level fluctuations together drove species diversification of slipper orchids (*Paphiopedilum*) in South-East Asia. *Molecular Ecology* 24: 2838–2855.

Guo, Y. Y., L. Q. Huang, Z. J. Liu, and X. Q. Wang. 2016. Promise and challenge of DNA barcoding in Venus Slipper (*Paphiopedilum*). *PLoS One* 11: e0146880.

Lang, K. Y., S. C. Chen, Y. B. Luo, and G. H. Zhu. 2006. Flora reipublicae popularis sinicae, 2nd ed., vol. 17, part II, 65. Science Press, Beijing, China.

Li, L., S. Zeng, F. Zheng, Z. Chen, K. Wu, J. Zhang, and J. Duan. 2010. Isolation and characterization of 10 polymorphic microsatellite loci in *Paphiopedilum concolor* (Batem.) Pfitzer (Orchidaceae) and cross-species amplification. *Hortscience* 45: 1286–1287.

Powell, W., G. C. Machray, and J. Provan. 1996. Polymorphism revealed by simple sequence repeats. *Trends in Plant Science* 1: 215–222.

Sun, Y. W., Y. J. Liao, Y. S. Hung, J. C. Chang, and J. M. Sung. 2011. Development of ITS sequence based SCAR markers for discrimination of *Paphiopedilum armeniacum*, *Paphiopedilum micranthum*, *Paphiopedilum delenatii* and their hybrids. *Scientia Horticulturae* 127: 405–410.

Thiel, T., W. Michalek, R. K. Varshney, and A. Graner. 2003. Exploiting EST databases for the development and characterization of gene-derived SSR-markers

in barley (*Hordeum vulgare* L.). *Theoretical and Applied Genetics* 106: 411–422.

Untergasser, A., I. Cutcutache, T. Koressaar, J. Ye, B. C. Faircloth, M. Remm, and S. G. Rozen. 2012. Primer3—New capabilities and interfaces. *Nucleic Acids Research* 40: e115.

Varshney, R. K., R. Sigmund, A. Borner, V. Korzun, N. Stein, M. E. Sorrells, P. Langridge, and A. Graner. 2005. Interspecific transferability and comparative mapping of barley EST-SSR markers in wheat, rye and rice. *Plant Science* 168: 195–202.

Yeh, F. C. 1997. Population genetic analysis of codominant and dominant markers and quantitative traits. *Belgian Journal of Botany* 129: 157.

Zeng, S., K. Wu, J. A. T. da Silva, J. Zhang, Z. Chen, N. Xia, and J. Duan. 2012. Asymbiotic seed germination, seedling development and reintroduction of *Paphiopedilum wardii* Sumerh., an endangered terrestrial orchid. *Scientia Horticulturae* 138: 198–209.

Zhang, F. P., J. L. Huang, and S. B. Zhang. 2016. Trait evolution in the slipper orchid *Paphiopedilum* (Orchidaceae) in China. *Plant Signaling and Behavior* 11: e1149668.

APPENDIX 1. Voucher specimen information for *Paphiopedilum* populations used in this study. Specimens are deposited at the Institute of Vegetables and Flowers (IVF), Chinese Academy of Agricultural Sciences, Beijing, China.

Species	Population	Voucher no.	Collection locality	Geographic coordinates	*N*
P. henryanum Braem[a]	Cultivated	Zhou IVF151008PH	CAAS, Beijing, China	39°57′43.94″N,116°19′35.65″E	1
P. henryanum[b]	Cultivated	Xu IVF170513PH	CAAS, Beijing, China	39°57′43.94″N,116°19′35.65″E	8
P. henryanum[b]	DY	Jia and Zhou DY130901PH	Danong, Yunnan, China	—	25
P. henryanum[b]	TYC	Jia and Zhou TYC130903PH	Taiyangchong, Yunnan, China	—	8
P. concolor (Bateman) Pfitzer[c]	Cultivated	Xu IVF171009PC	CAAS, Beijing, China	39°57′43.94″N,116°19′35.65″E	5
P. dianthum Tang & F. T. Wang[c]	Cultivated	Xu IVF171009PD	CAAS, Beijing, China	39°57′43.94″N,116°19′35.65″E	5
P. hirsutissimum (Lindl. ex Hook. f.) Stein	Cultivated	Xu IVF171009PVe	CAAS, Beijing, China	39°57′43.94″N,116°19′35.65″E	5
P. micranthum Tang & F. T. Wang[c]	Cultivated	Xu IVF171009PM	CAAS, Beijing, China	39°57′43.94″N,116°19′35.65″E	5
P. venustum (Sims) Pfitzer[c]	Cultivated	Xu IVF171009PVe	CAAS, Beijing, China	39°57′43.94″N,116°19′35.65″E	5
P. villosum (Lindl.) Stein[c]	Cultivated	Xu IVF171009PVi	CAAS, Beijing, China	39°57′43.94″N,116°19′35.65″E	5
P. wardii Summerh.[c]	Cultivated	Xu IVF171009PW	CAAS, Beijing, China	39°57′43.94″N,116°19′35.65″E	5

Note: CAAS = Chinese Academy of Agricultural Sciences; *N* = number of individuals sampled.
[a]Samples used for cDNA library construction.
[b]Samples used for initial PCR amplification trials and detailed evaluation for polymorphisms.
[c]Samples used for transferability test.