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Fourteen additional microsatellite markers for *Mussaenda pubescens* and cross-species amplification

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Introduction

Mussaenda pubescens Ait. f. is a species with stigma-dimorphism and functional dioecy, with yellow flowers and well-developed petaloid calyx-lobes, which is widespread in southern Chinese provinces Fujian, Guangdong, Guangxi, Hainan, Hunan, Jiangxi, Taiwan and Zhejiang (Li *et al.* 2010). A new variety *M. pubescens* var. *alba*, with white, stout corolla and much reduced or even lack of the petaloid calyx lobes which is different from typical *M. pubescens*, is also widely distributed in southern China (Deng and Zhang 2004). However, the two varieties never grow together. *M. pubescens* grows in more open places than *M. pubescens* var. *alba*, and the latter usually grows together in more dense habitats with other congeneric species with yellow flowers, such as *M. shikokiana*, *M. kwangtungensis* and *M. erosa*. Samples with intermediate morphologies between *M. pubescens* var. *alba*, and the sympatric *M. shikokiana* and *M. kwangtungensis* have been observed and natural hybrids have been identified (Shi Chen unpublished data). Microsatellites have proven to be efficient genetic markers for species delimitation, phylogenetic reconstruction and detection of hybrids (Xu *et al.* 2004; Barkley *et al.* 2005; Zhan *et al.* 2008). Nineteen microsatellite markers for *M. pubescens* have been developed previously (Duan *et al.* 2012), but they could not provide sufficient variation in population genetic structure analyses and in detecting interspecific hybridization in *Mussaenda* due to low polymorphisms. Here, we report 14 additional highly polymorphic microsatellite markers and expect them to provide additional tools for evaluating the intraspecific and interspecific population genetic structure and detecting the potential hybrids.

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Materials and methods

Genomic DNA of *M. pubescens* was extracted from silica gel-dried-leaf tissue of one individual in JM_MP (table 1) population following the cetyltrimethyl ammonium bromide (CTAB) method (Doyle 1991). Magnetic beads enrichment methods were used following Duan *et al.* (2012). Further, biotinylated oligonucleotides, $(AC)_{15}$, $(TC)_{15}$, $(CAA)_{15}$ were applied for enriching the appropriate segments of microsatellite loci separately. Three hundred and twenty-three clones were sequenced (Invitrogen, Guangzhou, China) and 105 primer pairs were designed by the software Primer Premier ver. 5.0 (Clarke and Gorley 2001). Six individuals of *M. pubescens* from DB_MP population were chosen to test the effectiveness and the annealing temperature for each primer pairs except AC_{30}, AC_{64}, CT_{142}, CT_{135}, CT_{99} and CT_{91} which annealed at 60–53°C (Piraee and Vining 2002). Forty-eight primer pairs could be amplified successfully. In addition, eight different individuals of this species belonging to three populations (table 1) were used to screen the polymorphisms by resolving on a 6% denaturing polyacrylamide gel. PCR was carried out under the same conditions as described in Duan *et al.* (2012). Fourteen SSR loci were detected polymorphic, each of the forward chains was fluorescent labelled (FAM, ROX, HEX or TAMRA, Invitrogen, Guangzhou, China) (table 2). Their polymorphisms were assessed using 169 individuals from three *M. pubescens* populations (DB_MP, TK_MP, LJ_MP), two *M. pubescens* var. *alba* populations (DQS_MPA, QFS_MPA), one population of *M. caudatiloba* (LR_MC), one population of *M. kwangtungensis* (NKS_MK), and one population of *M. hirsutula* (HN_MH). The detailed information is showed in table 1. The PCR was performed in a 10 μL mixture containing 5 μL Master Mix (Tiangen, Guangzhou, China), 0.2 μM of each primer pair, 3.6 μL deionized water, and 30–50 ng of genomic DNA. Cycling conditions were 94°C for 4 min, then

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Table 1. Sample information for *Mussaenda* species used in this research.

	Code	Location	Latitude	Longitude	Individual no.	Voucher no.
Mussaenda pubescens	DB_MP	Dianbai, Guangdong	21°33′	111°01′	22	Duan T.T.631
	TK_MP	Tangkou, Jiangmen, Guangdong	21°49′	111°34′	21	Duan T.T.671
	LJ_MP	Luojing, Guangxi	25°01′	110°05′	20	Cao X. Y. 2012060613
M. pubescens var. *alba*	QFS_MPA	Mt. Qifen, Guangxi	25°05′	110°35′	16	Chen S.70
	DQS_MPA	Duqiaoshan, Guangxi	22°47′	110°35′	29	Duan T.T.54
M. caudatiloba	LR_MC	Longrui Natural Reserve, Guangxi	22°28′	107°12′	24	Duan T.T.25
M. kwangtungensis	NKS_MK	Mt. Nankun, Guangdong	23°38′	113°51′	28	Deng X.F.502
M. hirsutula	HN_MH	Qixianling, Hainan	18°43′	109°41′	9	Duan T.T.145
Total					169	

35 cycles at 94°C for 30 s, 30 s at annealing temperature, and 30 s at 72°C, with a final extension of 8 min at 72°C. Additionally, a touchdown PCR programme one was used for some primers with initial denaturation for 4 min at 94°C, 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, followed 7 cycles at decreasing annealing temperatures in decrements of 1°C per cycle, then followed by 94°C for 30 s, 30 s at 53°C, 30 s at 72°C, ended with an extra extension of 8 min at 72°C (table 2). The alleles were scanned on the ABI Prism 3100 Genetic Analyser (Invitrogen, Guangzhou, China) and determined by GeneMarker ver. 2.4.0 (SoftGenetics LLC, State College, USA).

The software Cervus ver. 3.0.3 (Kallnowski *et al.* 2007) was used for calculating the observed alleles per locus (N_a), observed (H_o) and expected (H_e) heterozygosities in each population. Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) between all the loci were analysed by using GenePop ver. 4.0.7 (Rousset 2008).

Table 2. Characteristics of 14 microsatellite loci identified in *M. pubescens*, all of which were polymorphic.

	Primer sequence (5′–3′)	Repeat	Size (bp)	T_a (°C)	GenBank accession no.
AC30	F: GAAAATCCAAGAAACACAT*	$(TG)_5TT(TG)_4$	435–463	Touchdown	KF740515
	R: GACAACTCACAAGCCACTC				
AC64	F: AGGTCGTCCATGATTATAG*	$(TC)_7$	335–379	Touchdown	KF740516
	R: CAGAAACCGTGTAGTAGTG				
CT48	F: CGGTAAAAAAAAGGATGGAGA*	$(CT)_{19}$	316–350	53	KF740517
	R: ATGGTATTGCGAGATGGAAAA				
CT113	F: AACATACAGACCCAAGCC*	$(GA)_9$	276–356	Touchdown	KF740518
	R: AAGCACCTACGAACTCCC				
CT17	F: CCACAAAAAAGTAAACGCATA**	$(TC)_6TT(TC)_4$	285–323	56	KF740519
	R: CTCCCCTCTCACTGTAGAGAG				
CT142	F: CACTGGAGAAGAAAAGCG**	$(CT)_{17}$	255–311	Touchdown	KF740520
	R: GCATGTGCATATACCCGA				
CT59	F: ATTCCAGACACTTACTCACAGC**	$(CT)_{11}$	251–299	56	KF740521
	R: TGCAAACATACTTGATCCTACC				
CT12	F: CAAACTCGCTTCAAAAAAGTGACCATT***	$(CT)_{10}$	223–249	56	KF740522
	R: CAAAAAGCCAACTAAGCTACGACCATG				
CT60	F: CCTATATACTTGGTCTTGTGGT***	$(TC)_{10}$	204–276	58	KF740523
	R: CAGAACTATCTTATCTGTTGCC				
CT135	F: CAAAGCAAAGGATAGTAGGA***	$(AG)_{22}$	181–257	Touchdown	KF740524
	R: GTTGACAGATGCTGGTAATG				
CAA92	F: GGAAAAGATGACGGTTTGG***	$(AG)_5AC(AG)_4$	180–244	58	KF740525
	R: TAGTGATAAGCACGCCTGG				
CT99	F: CGGGGAGGTCTTGAAAGA****	$(CT)_7$	194–208	Touchdown	KF740526
	R: AGAGGAGGAGGTGGCGAT				
CAA112	F: GCAGGCTAGCTATTTCTCC****	$(TTC)_6$	152–178	53	KF740527
	R: TGTTCCTTCCCTTTGTTTT				
CT91	F: TGCTGAATAAGAATAACCT****	$(GA)_{10}$	147–155	Touchdown	KF740528
	R: TTTGAGTGAATATGCTGTA				

*FAM labelled; **ROX labelled; ***HEX labelled; ****TAMRA labelled. Presented for each locus are the forward (F) and reverse (R) primer sequences, repeat motif, size of the original fragment (bp), annealing temperature (T_a), and GenBank accession number.

Results and discussion

Forty-eight pairs of primers could be successfully amplified. Out of which fourteen of them showed polymorphism on the 6% denaturing polyacrylamide gel, while the others produced only one or multiple bands. Each of these 14 primers' forward chain was labelled by fluorescent dyes (table 2). All the primers could be amplified successfully in the four species and the variety. The allele size ranged from 147 to 463 bp, (table 2). The number of alleles ranged from 2 to 17, and the expected and observed heterozygosity varied from 0.000 to 0.950 and 0.000 to 0.942, respectively (table 3). When focussing on within-species and between-species polymorphism, the maximum number of alleles per locus was 17, 15, 17, 14 and 10 for LJ_MP, DQS_MPA, LR_MC, NKS_MK and HN_MH, respectively. The mean number of alleles per locus was 6.5, 5.8, 5.9, 6.3, 4.4, 8.4, 6.6 and 4.9 for DB_MP, TK_MP, LJ_MP, DQS_MPA, LR_MC, NKS_MK and HN_MH populations, respectively (table 3). The observed heterozygosities were 0.486, 0.439, 0.480, 0.377, 0.482, 0.490, 0.503, 0.382, respectively (table 3). The expected heterozygosities were 0.521, 0.466, 0.513, 0.361, 0.511, 0.583, 0.534, 0.590, respectively (table 3). Few loci showed significant deviation from HWE except in two populations (DQS_MPA and LR_MC) (table 3), and no LD was found among the loci, indicating their independence.

The genus *Mussaenda* possesses considerable diversity in sexual systems, providing great opportunities for studying population genetic differentiation patterns, speciation and sexual system evolution (Deng 2007Chen *et al.* 2013).

Table 3. Results of initial primer screening in populations of *Mussaenda* species.

	DB_MP (22 individuals)			TK_MP (21 individuals)			LJ_MP (20 individuals)			DQS_MPA (29 individuals)		
Locus	N_a	H_o	H_e	N_a	H_o	H_e	N_a	H_o	H_e	N_a	H_o	H_e
AC30	4	0.250	0.273	4	0.238	0.459*	4	0.450	0.545	1	0.000	0.000
AC64	3	0.182	0.280	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
CT48	12	0.909	0.859	12	0.810	0.870	13	0.800	0.899	15	0.655	0.885*
CT113	10	0.500	0.561	7	0.381	0.417	17	0.950	0.942	6	0.222	0.837*
CT17	9	0.773	0.803	7	0.476	0.513	5	0.789	0.747	8	0.759	0.750
CT142	10	0.864	0.854	8	0.762	0.787	9	0.700	0.718	10	0.724	0.817
CT59	6	0.682	0.572	4	0.619	0.632	3	0.200	0.415*	5	0.414	0.664*
CT12	4	0.619	0.659	4	0.905	0.693*	5	0.800	0.627	7	0.741	0.612
CT60	11	0.167	0.903*	16	0.857	0.941	10	0.421	0.872*	10	0.586	0.733*
CT135	11	0.909	0.822	8	0.619	0.717	4	0.750	0.668	15	0.621	0.751
CAA92	3	0.091	0.090	2	0.143	0.136	4	0.200	0.191	3	0.138	0.133
CT99	4	0.500	0.690	4	0.286	0.437	3	0.400	0.581	4	0.207	0.652*
CAA112	2	0.318	0.274	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
CT91	2	0.045	0.045	3	0.048	0.138	4	0.250	0.233	2	0.207	0.189
Mean	6.5	0.486	0.522	5.8	0.439	0.466	5.9	0.479	0.513	6.3	0.377	0.361

	QFS_MPA (16 individuals)			LR_MC (24 individuals)			NKS_MK (32 individuals)			HN_MH (9 individuals)		
Locus	N_a	H_o	H_e	N_a	H_o	H_e	N_a	H_o	H_e	N_a	H_o	H_e
AC30	2	0.438	0.466	2	0.042	0.042	4	0.219	0.306	1	0.000	0.000
AC64	1	0.000	0.000	9	0.667	0.757	1	0.000	0.000	4	0.000	0.733
CT48	7	0.625	0.752	6	0.708	0.637	10	0.656	0.734	7	0.556	0.686
CT113	8	0.875	0.732	12	0.833	0.868	9	0.563	0.804*	10	0.667	0.882
CT17	8	0.813	0.790	7	0.458	0.714*	10	0.656	0.799	6	0.556	0.745
CT142	6	0.688	0.752	17	0.542	0.907*	10	0.656	0.735	6	0.333	0.817
CT59	2	0.563	0.498	18	0.833	0.920	9	0.625	0.822*	4	0.333	0.529
CT12	3	0.375	0.486	2	0.250	0.496*	5	0.742	0.687	5	0.889	0.752
CT60	7	0.688	0.708	11	0.792	0.751	7	0.688	0.790	7	0.667	0.894
CT135	8	0.875	0.851	17	0.750	0.915*	14	0.773	0.905	3	0.125	0.608
CAA92	2	0.125	0.121	6	0.217	0.414*	5	0.781	0.746	8	0.556	0.830
CT99	4	0.250	0.629*	4	0.143	0.610*	4	0.438	0.458	5	0.556	0.673
CAA112	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	2	0.111	0.111
CT91	3	0.438	0.486	5	0.625	0.686	3	0.250	0.250	1	0.000	0.000
Mean	4.4	0.482	0.511	8.4	0.490	0.583	6.6	0.503	0.534	4.9	0.382	0.590

*Significant deviation from HWE ($P < 0.05$). The *M. hirsutula* population (HN_MH) did not test HWE for few individuals. N_a, number of alleles, H_o, observed heterozygosity, H_e, expected heterozygosity. DB_MP, *M. pubescens* from Dianbai, Guangdong; TK_MP, *M. pubescens* from Tangkou, Jiangmen, Guangdong; LJ_MP, *M. pubescens* from Luojing, Guangxi; DQS_MPA, *M. pubescens* var. *alba* from Duqiaoshan, Guangxi; QFS_MPA, *M. pubescens* var. *alba* from Mt. Qifen, Guangxi; LR_MC, *M. caudaliloba* from Longrui Nature Reserve, Guangxi; NKS_MK, *M. kwangtungensis* from Nankunshan, Guangdong; HN_MH, *M. hirsuluta* from Qixianling, Hainan.

Microsatellite markers have been proven to be valuable tools for analysing gene flow and hybridization (Zapiola and Mallory-Smith 2012). Its utilities in studying the genetic diversity, population structure and phylogenetic reconstruction are demonstrated by Aradhya *et al.* (2013) who developed and successfully applied 18 pairs of microsatellite markers in *Vitis*. Microsatellite markers have also been successfully used in elucidating the relationship between phenotypic plasticity and local adaptation (Frazer and Russello 2013). However, available SSR markers are limited to what we reported previously (Duan *et al.* 2012), which could not supply a high solution in our endeavour in phylogenetic reconstruction and species delimitation in the genus. Thus, these 14 additional polymorphic microsatellite primers will facilitate our future studies in phylogenetic reconstruction of the genus, in gene flow analyses and hybridization detection among sympatric species, and in elucidating the population genetic differentiation patterns between the two ecological races, viz., the yellow-flowered typical *M. pubescens* and the white-flowered *M. pubescens* var. *alba.*

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References

Aradhya M., Wang Y., Walker M. A., Prins B. H., Koehmstedt A. M., Velasco D. *et al.* 2013 Genetic diversity, structure, and patterns of differentiation in the genus *Vitis*. *Plant Syst. Evol.* **299**, 317–330.

Barkley N. A., Newman M. L., Wang M. L., Hotchkiss M. W. and Pederson G. A. 2005 Assessment of the genetic diversity and phylogenetic relationships of a temperate bamboo collection by using transferred EST–SSR markers. *Genome* **48**, 731–737.

Chen S., Luo Z. L. and Zhang D. X. 2013 Self–pollination in buds and homostyly in *Mussaenda shikokiana* (Rubiaceae), a monomorphic species in a distylous clade. *J. Syst. Evol.* **51**, 731–742.

Clarke K. R. and Gorley R. N. 2001 *Primer v5: User Manual/Tutorial*. Primer-E Ltd., Plymouth, UK.

Deng X. F. 2007 Taxonomic revision and phylogenetic analysis of breeding system evolution of *Mussaenda* L. (Rubiaceae) in China. Ph.D. dissertation. South China Botanical Garden, the Chinese Academy of Sciences, Guangzhou (in Chinese).

Deng X. F. and Zhang D. X. 2004 A new variety of *Mussaenda*. *J. Trop. Subtrop. Bot.* **12**, 476.

Doyle J. J. 1991 DNA protocols for plants. In: *Molecular techniques in taxonomy* (eds. G. M. Hewitt and A. Johnston) pp. 283–293, Springer-Verlag, Berlin, Germany.

Duan T. T., Gong W. and Zhang D. X. 2012 Development of microsatellite markers from *Mussaenda pubescens* (Rubicaeae). *Am. J. Bot.* **99**, e437–e439.

Frazer K. K. and Russello M. A. 2013 Lack of parallel genetic patterns underlying the repeated ecological divergence of beach and stream-spawning kokanee salmon. *J. Mol. Biol.* **26**, 2601–2621.

Kallnowski S. T., Taper M. L. and Marshall T. C. 2007 Revising how the computer program Cervus accommodates genotyping error increases success in paternity assignment. *Mol. Ecol.* **16**, 1099–1106.

Li A. M., Wu X. Q., Zhang D. X. and Barrett S. C. H. 2010 Cryptic dioecy in *Mussaenda pubescens* (Rubiaceae): a species with stigma-height dimorphism. *Ann. Bot.* **106**, 521–531.

Zapiola M. L. and Mallory-Smith C. A. 2012 Crossing the divide: gene flow produces intergeneric hybrid in feral transgenic creeping bentgrass population. *Mol. Ecol.* **21**, 4672–4680.

Piraee M. and Vining L. C. 2002 Use of degenerate primers and touchdown PCR to amplify a halogenase gene fragment from *Streptomyces venezuelae* ISP5230. *J. Ind. Microbiol. Biot.* **29**, 1–5.

Rousset F. 2008 Genepop'007: A complete re-implementation of the Genepop software for Windows and Linux. *Mol. Ecol. Res.* **8**, 103–106.

Xu Y., Ma R. C., Xie H., Liu J. T. and Cao M. Q. 2004 Development of SSR markers for the phylogenetic analysis of almond trees from China and the Mediterranean region. *Genome* **47**, 1091–1104.

Zhan Q. W., Zhang T. Z., Wang B. H. and Li J. Q. 2008 Diversity comparison and phylogenetic relationships of *S. bicolor* and *S. sudanense* as revealed by SSR markers. *Plant Sci.* **174**, 9–16.

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