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Short Communication

A new set of microsatellite loci for *Cattleya walkeriana* Gardner, an endangered tropical orchid species and its transferability to *Cattleya loddigesii* Lindl. and *Cattleya nobilior* Reichenbach

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Abstract

In this study, we isolate and analyse a new set of microsatellite loci for *Cattleya walkeriana*. Twenty-two primer pairs were screened for *C. walkeriana* ($n = 32$) and assessed for their transferability to *Cattleya loddigesii* ($n = 12$) and *Cattleya nobilior* ($n = 06$). All loci amplified for *C. walkeriana*; however, for *C. loddigesii* and *C. nobilior*, four and five primers, respectively, did not present amplification. The polymorphic loci presented between 2 and 13 alleles per locus for both *C. walkeriana* and *C. loddigesii*, with respective averages of 5.1 and 4.2. For *C. nobilior*, we found between two and five alleles per locus, with an average of 2.6. For *C. walkeriana*, observed heterozygosity varied from 0.100 to 0.966, whereas expected heterozygosity ranged from 0.097 to 0.900. The observed and expected heterozygosity for *C. loddigesii* and *C. nobilior* were also estimated. We found no significant linkage disequilibrium between any pair of loci, and evidence of null alleles at four loci (Cw16, Cw24, Cw30 and Cw31) for *C. walkeriana*. The combined power to exclude the first parent and combined non-exclusion probability of identity were 0.999 and 2.3×10^{-20}, respectively. These new loci can be used in studies of germplasm resources, and assessments of genotypic and genetic diversity and population structure, thus improving the accuracy of such analyses and their applicability in the conservation and protection of these endangered species.

Keywords: Brazilian orchid, conservation genetics, hybridization, population genetics, tropical orchid species

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Introduction

Accurate identification of genetic diversity within germplasm collections is essential for establishing and managing appropriate breeding programmes. The characterization of germplasm and analyses of population and conservation genetics based on molecular markers have gained importance due to the speed and quality of the generated data (de Vicente *et al.*, 2005; Govindaraj *et al.*, 2015). Microsatellites (SSRs) are highly informative molecular markers (Oliveira *et al.*, 2006; Vieira *et al.*, 2016) that have been developed for many plant species and are currently used in molecular breeding, germplasm evaluation, genetic diversity, genome mapping, hybridization and evolutionary studies (Gupta and Varshney, 2000; Govindaraj *et al.*, 2015; Tambarussi *et al.*, 2017).

In general, only a few sets of microsatellite markers are described in the literature for many species, despite the advantages they offer. Thus, there is a clear need to expand the available sets of these highly informative genetic markers, which can inform the effective management of species, improve germplasm resources, accelerate breeding programmes (Bajay *et al.*, 2011; Amorim *et al.*, 2012), and produce more accurate results in population genetics studies. As such, Tambarussi *et al.* (2016) developed eight microsatellite markers for *Cattleya walkeriana* for both commercial and conservation interests and new studies have been developed using these markers. However, to improve accuracy and increase the pool of available microsatellites, the present study provides 22 new polymorphic loci for future genetic studies on these endemic and endangered Brazilian orchids.

Experimental

Total genomic DNA was extracted from fresh leaves collected from a single plant of *C. walkeriana* (voucher number: 23052) from the Orchid Germplasm Collection of the Genetics Department (ESALQ/USP), University of São Paulo, Piracicaba, São Paulo, Brazil, using the protocol described by Doyle and Doyle (1990). A microsatellite-enriched genomic library was constructed following the protocol of Billotte *et al.* (1999), with the same criteria as that described in Tambarussi *et al.* (2016). Thirty-two specimens of *C. walkeriana* were analysed (voucher numbers: 23052a, 23052b, 23055, 2663, 85, 129RP, 100, 214, 907d, 905, 906, 882, 879, 887, 136RP, 1628, 12260, 132RP, 89, 128RP, 23189, 2409, 135RP, 1627, 907, 130RP, 88, 23207, 23193, 23068, 125RP and 2709). We also genotyped 12 accessions of *Cattleya loddigesii* (voucher numbers: 31077, 31073, 33626, 34026, 34029, 34030, 34033, 34034, 34044, 34046, 34050 and 34060) and six accessions of *Cattleya nobilior* (voucher numbers: 01, 02, 2354d, 30982, 30999 and 5652) to test the transferability of the microsatellite primers. All specimens of the three-species come from different populations.

Polymerase chain reactions (PCR) were performed in a final volume of 10 µl, using 5 µl of HotStarTaq Master Mix 2× (QIAGEN, Hilden, Germany), with 8 µM of M13 tail and reverse primers, 2 µM of forward primer according to Schuelke (2000) and 50 ng/µl of DNA. PCR cycling conditions were: initial denaturation at 95°C for 5 min, followed by 30 amplification cycles (95°C for 1 min, 1 min at the specific annealing temperature of each primer pair (online Table S1), 72°C for 1 min) and a final elongation step at 72°C for 7 min. Subsequently, 1 µl of the PCR product of each set of primers was added to 10 µl solution of formamide and GeneScan LIZ 500 dye Size Standard following the manufacturer's instructions. After solution denaturation at 95°C for 5 min, the PCR products were placed on an ABI 3130xl DNA analyser (Applied Biosystems) for automated capillary electrophoresis. For data analysis, we used the software GeneMapper v.5 (Applied Biosystems).

Genetic diversity was determined based on the number of alleles per locus (k) and observed (H_o) and expected (H_e) heterozygosity for each locus, and as an average across all loci. To test for linkage disequilibrium between pairwise loci, we used 1000 Monte Carlo permutations (alleles among individuals) and a Bonferroni correction (95%, $\alpha = 0.05$). These estimates were calculated using the FSTAT version 2.9.3.2 program (Goudet, 1995). Null allele frequencies for each locus was estimated using the program Micro-Checker 2.2.3 (van Oosterhout *et al.*, 2006), and verified using a Maximum-Likelihood approach implemented in the INEST version 2.1 program (Chybicki and Burczyk, 2009). The power to exclude the first parent (when none of the relatives are known) ($P_{1\circ\text{parent}}$) and allelic combined non-exclusion probability of identity (I) were estimated using Cervus 3.0.3 (Marshall *et al.*, 1998).

Discussion

All primer pairs amplified for *C. walkeriana*, but four and five primers showed no amplification for *C. loddigesii* and *C. nobilior*, respectively. We found 118 alleles (average of 5.1) for *C. walkeriana*, 73 alleles (average of 4.2) for *C. loddigesii*, and we identified 45 alleles (average of 2.6) for *C. nobilior* (Table 1; online Table S2). For *C. walkeriana*, H_o and H_e ranged from 0.100 to 0.966 and from 0.095 to 0.900, respectively. For *C. loddigesii* and *C. nobilior*, the average H_o was 0.479 and 0.531, and H_e was 0.562 and 0.478, respectively (Table 1; online Table S2).

Analysis using the Micro-Checker software revealed null alleles at four loci (Cw16, Cw24, Cw30 and Cw31) for *C. walkeriana*. However, due to the small sample size for *C. lodigesii* and *C. nobilior*, Micro-Checker could not perform the analysis. However, null alleles were not observed

Table 1. Results of initial primer screening in specimens of *Cattleya walkeriana*

	C. walkeriana ($n = 32$)					
Locus	k	H_o	H_e	$P_{1\circ parent}$	I	P_{Null}
Cw10	7	0.500	0.637	0.749	0.172	0.110
Cw11	2	0.100	0.097	0.799	0.212	0.000
Cw12	4	0.679	0.499	0.740	0.150	−0.255
Cw13	13	0.896	0.878	0.658	0.104	−0.026
Cw14	2	0.250	0.348	0.813	0.226	0.105
Cw15	1	NE	NE	NE	NE	NE
Cw16	11	0.408	0.858	0.734	0.148	0.2454*
Cw17	1	NE	NE	NE	NE	0.000
Cw18	4	0.167	0.833	0.549	0.061	0.000
Cw19	3	0.166	0.633	0.592	0.074	0.000
Cw20	5	0.125	0.699	0.428	0.032	0.000
Cw21	13	0.708	0.900	0.320	0.016	0.000
Cw22	11	0.800	0.896	0.711	0.131	0.043
Cw23	1	NE	NE	NE	NE	NE
Cw24	4	0.087	0.697	0.660	0.106	0.396*
Cw25	4	0.445	0.674	0.576	0.070	0.000
Cw26	4	0.444	0.546	0.638	0.096	0.000
Cw27	6	0.125	0.786	0.613	0.085	0.366
Cw28	4	0.625	0.459	0.852	0.315	−0.385
Cw29	2	0.966	0.500	0.517	0.051	−0.811
Cw30	12	0.607	0.870	NE	NE	0.153*
Cw31	3	0.250	0.460	0.749	0.172	0.214*
Mean	5.1	0.439	0.533	0.999[a]	2.3×10^{-20}[b]	
SD	3.9	0.278	0.311			
Total	118	–	–			

H_e, expected heterozygosity; H_o, observed heterozygosity; n, sample size for each population; k, the number of alleles per locus. *P_{Null}, van Oosterhout estimate for the frequency of null alleles at each locus. NE, not estimated. $P_{1\circ parent}$ is the power to exclude the first parent (when no parent is known). I, combined non-exclusion probability of identity.
[a]Combined exclusion probability.
[b]Combined estimation.

at any locus for the three-studied species using a Maximum-Likelihood approach. No linkage disequilibrium was detected in any pair of loci. Only one locus was deemed unsuitable for *C. walkeriana* due to low polymorphism or lack of amplification, while for *C. lodigesii* and *C. nobilior*, eight and 10 loci, respectively, are unsuitable (Table 1; online Table S2). All other loci are appropriate for genetics studies on these orchid species. The $P_{1\circ parent}$ (0.999) and I (2.3×10^{-20}) values indicate that the use of these loci are suitable for future parentage studies. The combined non-exclusion probability of identity (I) supports the use of these loci for identity analyses of these *Cattleya* species (Table 1; online Table S2).

These SSRs loci are suitable for genetic studies and can assist the investigation of possible interspecific crosses between species. They can also be used to support *in situ* and *ex situ* conservations, breeding programmes, genetic diversity and structure, mating system and gene flow of *C. walkeriana* and their related species.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S1479262117000193.

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