Microsatellite Primers for Two Threatened Orchids in Florida: Encyclia tampensis and Cyrtopodium punctatum (Orchidaceae)

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PRIMER NOTE

MICROSATELLITE PRIMERS FOR TWO THREATENED ORCHIDS IN FLORIDA: *ENCYCLIA TAMPENSIS* AND *CYRTOPODIUM PUNCTATUM* (ORCHIDACEAE)[1]

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- *Premise of the study:* The Million Orchid Project at Fairchild Tropical Botanic Garden is an initiative to propagate native orchids for reintroduction into Miami's urban landscapes. The aim of this study was to develop microsatellites for *Encyclia tampensis* and *Cyrtopodium punctatum* (Orchidaceae).
- *Methods and Results:* Ten microsatellites were developed for each species. For *E. tampensis* sampled from the natural population, allele numbers ranged from one to four, with an average observed heterozygosity (H_o) of 0.314 and average expected heterozygosity (H_e) of 0.281. For the individuals from cultivation, allele numbers ranged from one to six, with an average H_o of 0.35 and an average H_e of 0.224. For *C. punctatum*, allele numbers ranged from one to three, with an average H_o of 0.257 and an average H_e of 0.272.
- *Conclusions:* These microsatellites will be used to assess the genetic diversity of natural and cultivated populations with the intention of guiding genetic breeding under the Million Orchid Project.

Key words: commercially exploited plants; *Cyrtopodium punctatum*; *Encyclia tampensis*; microsatellites; native Florida orchids; Orchidaceae.

Fairchild Tropical Botanic Garden's Million Orchid Project (hereafter FTBG and MOP) is a special initiative to reintroduce millions of exploited and threatened native orchids into the urban landscapes of the Miami metropolitan area. The project focuses on reestablishment in schoolyards, roadways, and other public spaces. The focal species for this study, *Encyclia tampensis* (Lindl.) Small and *Cyrtopodium punctatum* (L.) Lindl. (Orchidaceae), were selected for reintroduction through the MOP based on their conservation importance, potential to be grown epiphytically in street trees, and their history of human exploitation. Through volunteer and student efforts, this program has propagated thousands of orchids for reintroduction. However, the genetic diversity of existing wild and cultivated populations of these species in the region is unknown.

Encyclia tampensis, also known as the butterfly orchid, is an epiphytic orchid native to southern Florida, Cuba, and The Bahamas (Luer, 1972). In southern Florida, it is found primarily in cypress swamps and tropical hammocks. The flowers, which look like butterflies, have varying amounts of red, green, and yellow hues in the petals (Subrahmanyam, 2008). The flowering period of *E. tampensis* in southern Florida is from May through August (Luer, 1972). In Florida, this species is considered commercially exploited according to a Florida state statute (Stat. No. 581.185 subsection 2(a); http://www.leg.state.fl.us/statutes/), suggesting that this species is subject to removal from the wild to be sold commercially.

Cyrtopodium punctatum, also known as the cigar or Florida cowhorn orchid, is a state-listed endangered orchid species native to southern Florida (Luer, 1972; Wunderlin et al., 2016). Wild populations of *C. punctatum* are also found in Mexico, the West Indies, and Central and South America (Romero-González and Fernández-Concha, 1999). *Cyrtopodium punctatum* is one of the only epiphytic species in the genus *Cyrtopodium* R. Br.,

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and in Florida is commonly found in cypress swamps and tropical hardwood hammocks (Chafin, 2000; Dutra et al., 2009). This orchid is characterized by bright yellow flowers splattered with dark burgundy spots and large pseudobulbs formed by former leaf bases. Populations have been degraded in southern Florida as a result of historic poaching, which has led to the legal protection of *C. punctatum* today (Stat. No. 581.185 subsection 2(a); http://www.leg.state.fl.us/statutes/).

The objectives of this study were to develop microsatellite markers for each species and assess the degree of genetic diversity of the existing populations at FTBG used in propagation efforts. These microsatellites were also tested for efficacy on two congeneric species for each focal species available at FTBG; congeneric species included *E. alata* (Bateman) Schltr., *Encyclia* hybrid 'Cindy' Das Orch. (*E. tampensis* × *E. alata*), *C. macrobulbon* (La Llave & Lex.) G. A. Romero-González & Carnevali f. *salvadorense* (Hamer & Garay) G. A. Romero-González & Carnevali, and *C. flavum* Link & Otto ex Rchb.

METHODS AND RESULTS

Leaf tissue was collected from one plant of *E. tampensis* (accession no. 160744) and one plant of *C. punctatum* (accession no. 160984) for next-generation sequencing; both individuals are located at FTBG (Coral Gables, Florida, USA). Due to the rarity of these species and potential for poaching, GPS coordinates are not given for any individual plants in this study. Photo vouchers for each individual are deposited in the FTBG Herbarium (Coral Gables, Florida, USA). Genomic DNA was extracted from lyophilized tissue using the DNeasy Plant Mini Kit (QIAGEN, Venlo, The Netherlands) at FTBG laboratories. The total DNA was sent to the Georgia Genomics Facility at the University of Georgia (Athens, Georgia, USA) for next-generation sequencing using Illumina HiSeq 2000 (Illumina, San Diego, California, USA). The resulting 100-bp Illumina sequences were screened using the PERL script program PAL_FINDER_v0.02.03 to identify potential microsatellite repeat elements among the reads (Castoe et al., 2012). No additional sequencing was done after the Illumina run. Microsatellite repeats were detected in one direction; if one was found, the sequence was sent automatically to Primer3 version 2.0.0 (Koressaar and Remm, 2007; Untergasser et al., 2012) for primer design. We only considered primer pairs that occurred 1–10 times among the reads. Primers were designed for sequences containing a microsatellite region and sufficient flanking sequence.

For *E. tampensis*, 48 loci containing microsatellite motifs with at least seven uninterrupted repeats were selected for initial primer design. For *C. punctatum*, 48 microsatellite regions with at least three motifs were identified. The different PCR primer pairs were manufactured at Integrated DNA Technologies (Coralville, Iowa, USA). These unlabeled primers were screened for unique products on the same individuals used for next-generation sequencing. The PCR products were prepared using 1 μL of genomic DNA, 11.5 μL of ddH_2O, 2.5 μL of TBT PAR (solution contains 750 mM trehalose [Sigma-Aldrich, St. Louis, Missouri, USA], 1 mg/mL nonacetylated bovine serum albumin [BSA; Sigma-Aldrich], 1% Tween-20 [Acros Organics, Geel, Belgium], and 8.5 mM Tris hydrochloride [pH 8.0; Fisher Scientific, Pittsburg, Pennsylvania, USA]), and a 10-μL mixture of the primer pairs (10 μM concentration) using a Dyad Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA). The PCR conditions were as follows: initial denaturation at 94°C for 3 min; followed by 32 cycles of 94°C for 1 min, annealing for 1 min (temperature varied by species), and 72°C for 2 min; and elongation at 72°C for 30 min. Annealing temperatures were 62°C for the *E. tampensis* primer mix and 58°C for the *C. punctatum* primer mix (Tables 1 and 2). The PCR products were scored for presence or absence of a single appropriately sized DNA fragment using gel electrophoresis on 2% agarose gel. Based on these criteria, 13 and 11 of the 48 potential loci for *E. tampensis* and *C. punctatum*, respectively, were selected for dye-labeling at Applied Biosystems (Foster City, California, USA). Fragment analysis was then used to further detect any primer pairs that did not produce the expected PCR product, and any locus that showed multiple and/or overlapping peaks was omitted. For each species, the 10 best novel microsatellite loci were selected for their repeatability, potential polymorphism, and capacity for multiplexing (Tables 1 and 2).

Under these PCR conditions, 10 primers were then tested for polymorphism on a total of 20 individuals of *E. tampensis* from natural and cultivated sources, as well as one individual of *E. alata* and one individual of *Encyclia* hybrid 'Cindy'. Sampling for the natural population at FTBG consisted of 14 individuals, and the cultivated population was represented by six individuals. Cultivated individuals were obtained from a commercial nursery in Miami-Dade County, Florida.

Ten primer pairs containing microsatellite regions were screened on 21 individuals of *C. punctatum*, two individuals of *C. macrobulbon*, and one individual of *C. flavum*. The PCR reactions consisted of 1 μL of genomic DNA, 14 μL of ddH_2O, and 10 μL of a primer mix; the total mixture included seven primer pairs: 12 μL of Y01, 24 μL of Y02, 36 μL of Y03, 24 μL of B01, 36 μL

Table 1. Characteristics of 10 microsatellite primer pairs developed for *Encyclia tampensis*.[a]

Locus[b]	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	Fluorescent dye[c]	GenBank accession no.
YE1	F: CTCAAACCACCTCCAACAGC R: GGCATTTAATTAAGAGACTAACCTCC	$(AATT)_7$	156	NED	KT36366
YE2	F: GCAAGTCTAGAATGAGATTTC TGGC R: CATCTGCTATTCAGTCTTTGTTCG	$(AAAG)_8$	196	NED	KT363662
YE3	F: TTAGTCGTTCATTCTCAGCCG R: AAATTCAGTTTGATCAGTGCCG	$(ATAC)_7$	208	NED	KT363663
YE4	F: CCATATCGAGATTGAATATCATCACC R: CACCGACTGTTATCTTCAACTGC	$(AAAT)_7$	265	NED	KT363664
YE5	F: GCAGGGATGAGGATTGTAAGG R: TTCAACAGAGTTCTCATCAGTTACC	$(AATG)_7$	483	NED	KT363665
GE1	F: GCCTCCTAAGATAATGCCTTCC R: AAATTGGGTTTGATTGAGGAGG	$(AATT)_7$	154	HEX	KT363666
GE2	F: ATGGGCGGTTTCTCATAACG R: TTCTTTACGCGTCATTCTTTGG	$(AAAT)_7$	193	HEX	KT363667
GE3	F: ATTGAATGGCCAAGGGACC R: GATGCCTTATCCCTCTTGGG	$(AATT)_7$	144	HEX	KT363668
BE1	F: CCACAATCATGCAAGTGATGC R: TTGTTGCACAGCCCTTGG	$(AAAT)_7$	169	6-FAM	KT363669
BE2	F: GTTGTTTGTAGATTGATTCTCTTTCATCC R: TTCATTAGCGATTTCTTCTCAAATGC	$(AAAT)_7$	232	6-FAM	KT363670

Note: T_a = annealing temperature.
[a]Annealing temperature was 62°C for all loci.
[b]Loci were named according to the color of the dyes used as follows: yellow (YE), green (GE), and blue (BE).
[c]5′-end dye labeled.

TABLE 2. Characteristics of 10 microsatellite primer pairs developed for *Cyrtopodium punctatum.*

Locus[a]	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	T_a (°C)	Fluorescent dye[b]	GenBank accession no.
Y01	F: GCTCCGAGATAATGCAGCG	$(TTC)_{15}$	181	58	NED	KT363671
	R: GAGCCTCCTCAGATAAATTCCC					
Y02	F: GGATAAAGCACCTGTAAGCAGC	$(TTC)_{16}$	254	58	NED	KT363672
	R: GACACTTCGAGCTGTCGTCC					
Y03	F: TGAGTGGTGTCCTAACCAACC	$(ATGG)_{11}$	311	58	NED	KT363673
	R: AAGGCCTTTACATTGAAGAATGG					
G01	F: GGTGGAGATGGATGATGGG	$(TTC)_{14}$	150	58	HEX	KT363674
	R: AAACTCTAATTCTCAACA CAGAGGG					
G02	F: CCCTAAGATCTACGTGGTGTCG	$(ATGG)_8$	233	58	HEX	KT363675
	R: CTCCTTGACCCAAGTCAGCC					
G03	F: CAGGTTTCCTCTCTCTTCG	$(TTC)_{16}$	309	58	HEX	KT363676
	R: CAATAATGGAGGATTGCGTGG					
B01	F: GGAAGAGCAACGAAAGAGGC	$(AATC)_7$	195	58	6-FAM	KT363677
	R: GCAATGCTAAGCACATAGACGG					
B02	F: TTTGTCATCGCTGCAAAGC	$(AGTG)_9$	299	54	6-FAM	KT363678
	R: GGAGTCGAAGCTGAAGGAGC					
B03	F: GAGCATTTGCATAAACATTGTGG	$(ATT)_{17}$	226	54	6-FAM	KT363679
	R: TGAACATCGTATGGTACTAATTTGAGG					
B04	F: TCAAGTTGGACAGTTGAGATGG	$(TTC)_{15}$	187	55	6-FAM	KT363680
	R: GGCGAAATTTCTGTGAGTTCC					

Note: T_a = annealing temperature.
[a] Loci were named according to the color of the dyes used as follows: yellow (Y), green (G), and blue (B).
[b] 5′-end dye labeled.

of G01, 36 µL of G02, and 36 µL of G03 (10 µM concentration), and PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) using a Dyad Peltier Thermal Cycler (Bio-Rad). As a result of interaction effects, three primer pairs—B02, B03, and B04—were run individually. Annealing temperature was 54°C for B02 and B03, and 55°C for B04 (Table 2).

The PCR products were prepared for fragment analysis in a reaction mixture of 1 µL of amplified DNA, 9.5 µL of Hi-Di Formamide (Applied Biosystems), and 0.5 µL of MapMarker (BioVentures, Murfreesboro, Tennessee, USA). The samples were sent to the Core DNA Facility at Florida International University (Miami, Florida, USA) for fragment analysis. Resulting chromatograms were visualized and scored using GeneMapper version 5.0 software (Applied Biosciences). Genotypic and allelic frequencies were obtained using Hardy–Weinberg equilibrium (HWE) Calculator for 3 Alleles (Emerson, 2010) to describe differences in allelic equilibrium assumptions. A χ^2 analysis was used to test for statistically significant deviations from HWE for the observed genotype frequencies using Microsoft Excel (Microsoft Corporation, Seattle, Washington, USA).

For each population, the total number of alleles, fragment length, and the observed (H_o) and expected heterozygosity (H_e) were calculated for each microsatellite region, and the average heterozygosities and test statistics for HWE were calculated for each species (Tables 3 and 4). In the natural population of *E. tampensis*, the number of alleles per locus varied from one to four, and three loci (YE3, GE1, and BE1) showed significant deviation from HWE (Table 3). In the cultivated population, the number of alleles per locus was greater, from one to six, and three loci (GE1, BE1, and BE2) showed significant deviation from HWE (Table 3). However, only the YE5 loci amplified six alleles; the other nine loci were composed of only one or two alleles. The average H_o and H_e for the natural population were 0.314 and 0.281, respectively (Table 3). For the cultivated population, average H_o was 0.35, higher than that of the natural population, with a lower average H_e of 0.224. For the closely related taxa, allele numbers ranged from one to three. For the congeneric *E. alata*, nine of the 10 loci amplified, while for the hybrid *Encyclia* 'Cindy' all 10 loci successfully amplified, and we detected a different allele than *E. tampensis* at the YE2 locus (Table 3).

Seven of the 10 primers tested for *C. punctatum* revealed polymorphism (Table 4). For the *C. punctatum* population at FTBG, the number of alleles per locus varied from one to three (Table 4). The average H_o was 0.257, and average H_e was 0.272. The H_o for three alleles (G01, B03, and B04) showed significant deviations from HWE (Table 4). For the congener *C. macrobulbon*, all 10 microsatellite regions amplified with up to four alleles, and for *C. flavum* nine microsatellites amplified a maximum of two alleles, and the majority of the loci were monomorphic.

TABLE 3. Genetic properties and descriptive statistics of the 10 novel microsatellites for *Encyclia tampensis* and two closely related taxa, *E. alata* and *Encyclia* hybrid 'Cindy' (*E. tampensis* × *E. alata*).

	Natural population (N = 14)			Cultivated population (N = 6)			*E. alata* and *Encyclia* 'Cindy' (N = 2)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
YE1	1	0	0	1	0	0	1	0	0
YE2	2	0.14	0.13	1	0	0	2	1	—
YE3	2	0[†]	0.13	1	0	0	2	0	0.38
YE4	1	0	0	1	0	0	1	0	0
YE5	4	0.57	0.68	6	0.83	0.81	1	0	0
GE1	2	1[†]	0.5	2	1[†]	0.5	2	1	0.5
GE2	4	0.29	0.53	2	0.67	0.44	3	1	0.63
GE3	1	0	0	1	0	0	1	0	0
BE1	2	1[†]	0.5	2	1[†]	0.05	3	1	0.5
BE2	2	0.14	0.34	2	0[†]	0.44	3	0.5	0.38
Average	2.1	0.314	0.281	1.9	0.35	0.224	1.9	0.45	0.265

Note: A = number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals sampled.
[†] Significant deviation from HWE ($P < 0.05$).

TABLE 4. Genetic properties and descriptive statistics of the 10 novel microsatellites for three species of *Cyrtopodium*.

	C. punctatum ($N = 21$)			*C. macrobulbon* ($N = 2$)			*C. flavum* ($N = 1$)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
Y01	1	0	0	2	0	0	1	0	—
Y02	2	0.57	0.48	2	0	0	1	0	—
Y03	2	0.62	0.66	2	0.25	0.2	1	0	—
G01	3	0.14†	0.29	3	0.25	0.2	2	1	—
G02	1	0	0	1	0	0	1	0	—
G03	1	0	0	1	0	0	1	0	—
B01	1	0	0	1	0	0	1	0	—
B02*	3	0.28	0.203	2	0.25	0.2	1	0	—
B03*	2	0.48†	0.25	4	1	0.25	2	1	—
B04*	2	0.48†	0.25	2	1	0.25	2	1	—
Average	1.8	0.257	0.272	2.0	0.275	0.11	1.2		

Note: A = number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals sampled.
*Locus was not included in primer mix.
†Significant deviation from HWE ($P < 0.05$).

CONCLUSIONS

Here we report 10 novel microsatellite loci each for two commercially exploited native Florida orchids, *E. tampensis* and *C. punctatum*. The microsatellites developed showed monomorphic and polymorphic differences in the two study populations, as well as some applicability for closely related taxa. Based on the number of alleles amplified and the average H_o values for *E. tampensis*, these results suggest that the cultivated population is more genetically diverse than the natural population at FTBG; however, this conclusion is given with caution, because our sampling effort (number of plants sampled) was not representative of the entire population, and only one locus showed amplification of six alleles in the cultivated population. Allele numbers for the other *Encyclia* species were comparable to that of the *E. tampensis* natural population. The genetic diversity of *C. punctatum* individuals at FTBG, on average, was not significantly different than the H_e (<2% difference). Because *C. punctatum* is an outcrossing species and the individuals sampled included several seedling volunteers, these findings indicate that an adequate amount of outcrossing is occurring in the garden population. Considering this evidence and the rarity of this species, this population may be an important source of diverse genetic material for diversifying the more vulnerable natural populations.

The resulting markers will be used to evaluate the genetic diversity, population demography, and potential gene flow of the remaining natural and cultivated populations in the region, with the intention of guiding the genetic breeding protocols for future reintroductions under the MOP. Specifically, we will assess the genetic diversity of all known individuals of *C. punctatum* and *E. tampensis* at FTBG. To ensure sufficiently diverse individuals are being selected for reintroduction, we will also screen offspring propagated from natural or artificial fruit set to be used for the MOP. More broadly, these microsatellite markers can be used to study genetic diversity of other populations within Miami and will be imperative in any future conservation efforts for these endangered orchid species.

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