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Short Communication

A set of variable plastid SSR markers for the genus *Cryptanthus* (Bromeliaceae)

Florian Krapp[1*], Geyner Alves dos Santos Cruz[2], Tina Wöhrmann[1], Ana Maria Benko-Iseppon[2] & Kurt Weising[1]

[1]Plant Molecular Systematics, Department of Sciences, University of Kassel, D-34132 Kassel, Germany
[2]Genetics Department, CCB, Universidade Federal de Pernambuco (UFPE), Av. Prof. Moraes Rego, 1235, 50.670-420, Recife, PE, Brazil
*Corresponding author e-mail: floriankrapp@gmx.de

The genus *Cryptanthus* (Bromeliaceae) is endemic to Brazil. Many of its currently recognized 66 species are narrow endemics that are threatened by habitat destruction. Molecular markers are needed to evaluate the extent and distribution of genetic diversity in rare *Cryptanthus* species, which would be a prerequisite for taking appropriate conservation measures. Here we describe the development of plastid microsatellite markers (cpSSRs) for *Cryptanthus*. PCR primers specific for 34 cpSSR loci in *Dyckia marnier-lapostollei* were initially tested for their functionality in *Cryptanthus schwackeanus*. PCR was successful for 29 loci, and 13 loci were shown to harbour extended stretches of mononucleotide repeats. Seven loci were further characterized by genotyping *Cryptanthus* samples at the level of populations and species, and six loci proved to be polymorphic among 30 individuals of each of the two endangered species *C. schwackeanus* and *C. warren-loosei*, respectively. All primers cross-amplified in other genera from three subfamilies of Bromeliaceae.

Keywords : Bromeliaceae; Bromelioideae; *Cryptanthus*; cpSSR; microsatellites; population genetics

The genus *Cryptanthus* Otto & A. Dietr. (Bromeliaceae) comprises 66 species that are endemic to Brazil. *Cryptanthus* species can be found in a variety of habitats, ranging from semi-humid locations in Atlantic Forests to the semi-arid Campos Rupestres, which form exposed "rocky fields" in the Cerrado and Caatinga biomes of eastern Brazil. The genus is distributed from Rio Grande do Norte in the north to Rio de Janeiro in the south, forming a centre of biodiversity in the states of Minas Gerais and Espírito Santo (Ramirez-Morillo, 1996; Ramirez-Morillo & Brown, 2001; Luther, 2008; Forzza et al., 2011). In general, *Cryptanthus* species have narrow distribution areas, and many of them are micro-endemics. Due to ongoing deforestation, 26 *Cryptanthus* species have become quite rare and are now included in the list of endangered species, such as the particularly vulnerable *C. schwackeanus* Mez (Martinelli et al., 2008). *Cryptanthus* species from the Campos Rupestres of Minas Gerais and Bahia are additionally threatened by mining, anthropogenic fires, and grazing. Here, the ongoing habitat destruction is compromising the survival of e.g. *C. warren-loosei* Leme, a micro-endemic species of that habitat in Bahia (Versieux et al., 2008).

The successful implementation of conservation measures for *Cryptanthus*

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requires some basic knowledge about the extent and distribution of genetic diversity within and among species. Such knowledge can be collected via appropriate population genetic surveys. Suitable molecular genetic marker systems are however needed for this purpose. Here we describe the development of a set of highly polymorphic chloroplast microsatellite markers (cpSSRs) for *Cryptanthus*, based on previously analyzed plastome sequences from another bromeliad, *Dyckia marnier-lapostollei* L.B.Sm. (Krapp et al., 2012).

Materials and methods

DNA was isolated from leaves of individual plants following Tel-Zur et al. (1999). Thirty-four primer pairs that flank cpSSR loci in *Dyckia marnier-lapostollei* were initially tested on DNA from a single accession of *C. schwackeanus*. PCRs were carried out in 10 µL volumes in a Biometra T-Gradient cycler, using the indirect fluorescence labelling procedure described by Schuelke (2000). Each assay contained approximately 1 ng of template DNA, 1x Mango-*Taq* reaction buffer (Bioline), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.04 µM forward primer carrying a 5'-M13 tail, 0.16 µM of M13 forward primer with fluorescent 5'-IRD700 modification, 0.16 µM unlabeled reverse primer, 0.5 µg/µl BSA and 0.05 U Mango-*Taq* DNA polymerase (Bioline). All loci were amplified using a standard PCR program with an initial denaturation at 80°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 52°C for 1 min and elongation at 65°C for 2 min. Final extension was performed at 65°C for 10 min. Samples were electrophoresed on denaturing 6% polyacrylamide gels in 1xTBE buffer, using an automated sequencer (Li-Cor 4300, Li-Cor Biosciences). Fragment sizes were determined by eye with the help of an external size standard, as outlined by Wöhrmann et al. (2012). Allele sizes were validated by repeated PCR amplification of selected samples. PCR products for one accession of *C. schwackeanus* were sequenced directly, using a Thermo Sequenase Primer Cycle Sequencing Kit (GE Healthcare) and an automated sequencer (Li-Cor 4300, Li-Cor Biosciences), following the protocols supplied by the manufacturers.

Results and discussion

Twenty-nine of the 34 loci showed distinct bands on agarose when tested with a single accession of *C. schwackeanus*. The presence and copy number of A/T mononucleotide repeats in the PCR products were validated by single nucleotide sequence analyses (Guicking et al., 2008). A/T repeats with N≥10 were found at 13 loci, which were further evaluated by amplifying an extended set of *Cryptanthus* individuals from three species, *C. schwackeanus*, *C. warren-loosei* and *C. sergipensis* I. Ramírez. Seven loci proved to be suitable for further study, based on the extent of variation within species and populations, the rate of successful PCR amplification, and the ease of sizing. Primer sequences, locus characteristics and GenBank accession numbers are shown in Table 1. These seven loci were further characterized by genotyping 30 individuals (three populations) of each of the two endangered species *C. schwackeanus* and *C. warren-loosei* as well as several samples from related species and genera. The results are summarized in Table 2. Six loci were polymorphic within *C. schwackeanus* with two or three alleles each, resulting in a total of eight different haplotypes. Three loci also showed polymorphisms within populations. Six loci were polymorphic in *C. warren-loosei*, with two or three alleles per locus combining into five different haplotypes. All six loci were polymorphic in at least one population. The seven cpSSR loci revealed between four and eight alleles within a total of five species of *Cryptanthus,* and all primers amplified well in ten other genera of Bromelioideae, five of Pitcairnioideae, and one of Puyoideae (Table 2).

Table 1 Characteristics of seven chloroplast microsatellite markers (cpSSRs) developed for *Cryptanthus* shown with GenBank accession numbers of the sequences of the locus in *C. schwackeanus* (sequenced for individual 01 of the population from Serra da Piedade). Primer sequences are derived from *Dyckia marnier-lapostollei* (Krapp et al., 2012)

Locus	Primer sequence (5'-3')	Position	Motif	Size (bp)	GenBank Accession No.
Crypt_cpSSR_01	fwd: 5'-CCTATTACAGAGATGGTGCG-3' rev: 5'-TTTCTCGTAAGACTGAGGGC-3'	*ycf*3 intron 2/exon 3 intronic/exonic	$(T)_9$	69	KC111433
Crypt_cpSSR_02	fwd: 5'-GTTCCCAGTAAGAACCAACC-3' rev: 5'-CTCAATAATTTCACATTTCC-3'	*rpo*C1 Intron intronic	$(T)_{13}$	104	KC111434
Crypt_cpSSR_03	fwd: 5'-TTGTTGTGGTATCTTTCGCC-3' rev: 5'-CAAGATTTCTCTGATACCCG-3'	*psb*B-*psb*T intergenic	$(T)_{10}$	64	KC111435
Crypt_cpSSR_04	fwd: 5'-TNAATCAATATGGCGAAGGC-3' rev: 5'-ATTCCCTCACGCTTGGCGCC-3'	*clp*P Intron 2 intronic	$(T)_{10}$	79	KC111436
Crypt_cpSSR_05	fwd: 5'-TTTTTGTTATGGGTATTCCC-3' rev: 5'-ACAAACAGAAAAGAGAGGGC-3'	*atp*F intron intronic	$(T)_{12}$	71	KC111437
Crypt_cpSSR_06	fwd: 5'-CTTCCATTTATCCATATCCC-3' rev: 5'-AAAATAAATCTGATTATGGG-3'	*rpl*16 Intron intronic	$(T)_{14}$	67	KC111438
Crypt_cpSSR_07	fwd: 5'-GTGGATTTATTTTTTGTCCC-3' rev: 5'-AGACCCCGGGCTCGAGGACG-3'	*rps*3-*rpl*22 intergenic	$(TA)_4(T)_{13}$	170	KC111439

Table 2. Observed allele sizes (bp) at seven chloroplast microsatellite loci in *C. schwackeanus* and *C. warren-loosei*, each represented by three populations with N=10. Allele numbers and allele size range among five different *Cryptanthus* species (*C. acaulis* Beer, *C. fosterianus* L.B. Sm., *C. microglazioui* I. Ramírez, *C. schwackeanus* and *C. warren-loosei* (between one and 30 individuals each) and cross-amplification in the subfamilies Pitcairnioideae, Bromelioideae (excluding *Cryptanthus*) and Puyoideae.

Locus	*C. schwackeanus*			*C. warren-loosei*			*Cryptanthus*	subfamily Pitcairni-oideae	subfamily Bromeli-oideae	*Puya*
	Serra da Piedade*	Serra da Calçada*	Serra da Brígida*	Jacobina*	Buraco do Possidônio (MCH)*	Guariba (MCH)*	allele number (size range in bp)			
Crypt_cpSSR_01	69, 71	69,71	69,71	80	75, 80	79, 80	8 (69-80)	2 (69-70)	5 (68-74)	1 (69)
Crypt_cpSSR_02	104	105	104	101	101, 104	101	6 (100-105)	3 (101-104)	5 (96-109)	1 (101)
Crypt_cpSSR_03	64	64	64	65	64, 65	63, 64	4 (61-65)	2 (63-64)	5 (62-69)	1 (64)
Crypt_cpSSR_04	79	80, 81	80	79	78, 79	78	5 (78-86)	3 (78-80)	5 (78-84)	1 (81)
Crypt_cpSSR_05	71, 72	72	73	68	68	68	4 (68-73)	4 (67-71)	4 (67-70)	1 (67)
Crypt_cpSSR_06	67	70	62	63	62, 63	63, 64	6 (61-70)	4 (60-65)	5 (60-65)	1 (63)
Crypt_cpSSR_07	170	172	176	170	169, 170	170	7 (161-176)	5 (156-163)	8 (161-179)	1 (159)

* Population (sampling location)

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