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ORIGINAL ARTICLE

Cross-amplification of nuclear microsatellite markers in two species of *Cryptanthus* Otto & A. Dietr. (Bromeliaceae)

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Abstract Thirty-eight nuclear microsatellite loci originally developed for *Aechmea caudata* Lindm., *Orthophytum ophiuroides* Louzada & Wand., *Pitcairnia albiflos* Herb., *Vriesea gigantea* (Gaud.) and *V. simplex* (Vell.) Beer were tested in *Cryptanthus burle-marxii* Leme and *C. zonatus* (Vis.) Vis. Of the 38 loci tested, 13 were polymorphic. Ten polymorphic microsatellite loci were selected to be amplified and genotyped in one population each of *C. burle-marxii* and *C. zonatus.* The observed and expected heterozygosity per locus in the *C. burle-marxii* population ranged from 0.050 to 0.850 and 0.050 to 0.770, respectively. In *C. zonatus*, the observed and expected heterozygosity per locus ranged from 0.167 to 0.846 and 0.290 to 0.692, respectively. The *O. ophiuroides* locus Op52 for the *C. zonatus* population and *P. albiflos* locus PaC05 for the two species showed significant departure from HWE. These ten polymorphic loci tested will be used to assess the genetic diversity and structure of the two species of *Cryptanthus.*

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Introduction

The genus *Cryptanthus* Otto & A. Dietr. (Bromeliaceae) comprises 78 species restricted to Brazil, where they occur in the Atlantic Forest, "Caatinga" and "Cerrado" (Forzza et al. 2016). These species belong to the Bromelioideae subfamily and are terrestrial and/or saxicolous herbs (Ramírez-Morillo 1996). Traditionally *Cryptanthus* has been recognized as sister group to the genus *Orthophytum* (Ramírez-Morillo 1996), but there is recent evidence that both genera are not monophyletic (Louzada et al. 2014).

Cryptanthus burle-marxii Leme and *C. zonatus* (Vis.) Vis. are terrestrial plants restricted to the northern portion of the Atlantic Forest in northeastern Brazil (Forzza et al. 2016). *Cryptanthus burle-marxii* is distributed in areas of coastal forests known as *Restinga*, while *C. zonatus* can be found within wet forests several kilometers from the coast. *Cryptanthus zonatus* also occurs in elevated islands of wet forest surrounded by "Caatinga", which are called *Brejos de Altitude* in Brazil (Leme and Siqueira Filho 2006). The two species are classified as threatened, specifically as Vulnerable (VU: Vulnerable category of the IUCN) by the "Livro Vermelho da Flora do Brasil" (Red List of the Brazilian Flora; Forzza et al. 2013).

These two species are considered a species complex due to overlapping morphological characters (Ramírez-Morillo 1996; Versieux et al. 2013). This complex previously included *Cryptanthus fosterianus* L.B. Smith before/until it was synonymized under *C. zonatus* in a recent morphological analysis (Alves and Marcucci 2015). However, a strictly morphological approach is insufficient for confident

species delimitation in this complex. Molecular markers such as microsatellites (simple sequence repeats, SSRs) can be used in addition to morphological characters to improve confidence in taxonomy (Caddah et al. 2012). Microsatellites can be obtained by PCR (polymerase chain reaction) amplification using specific primers (Faleiro 2007). While developing custom primers for new SSRs can be cost prohibitive (Oliveira et al. 2006), it is possible to use primers previously developed for closely related species in a process called cross-amplification (Oliveira et al. 2006).

Previous studies involving the development and cross-amplification of SSRs markers in Bromeliaceae have primarily focused on species of the Tillandsioideae (Boneh et al. 2003; Palma-Silva et al. 2007; Lavor et al. 2013; Neri et al. 2015) and Pitcairnioideae subfamilies (Sarthou et al. 2003; Paggi et al. 2008; Krapp et al. 2012; Miranda et al. 2012; Wöhrmann et al. 2012, 2013; Zanella et al. 2012; Hmeljevski et al. 2013). Few studies have focused on the species in the subfamily Bromelioideae (Goetze et al. 2013; Krapp et al. 2013; Aoki-Gonçalves et al. 2014). There are no published SSRs markers developed specifically for the genus *Cryptanthus*, only studies with cross-amplification of chloroplast microsatellite markers, where primers specifically developed for *Dyckia marnier-lapostollei* L.B.Sm. were tested in *Cryptanthus schwackeanus* Mez and *C. warren-loosei* Leme (Krapp et al. 2013). Cross-amplification of nuclear SSRs has never been tested within the genus *Cryptanthus*.

Here we report the cross-amplification of nuclear microsatellite markers of *Aechmea caudata* Lindm. (Bromelioideae subfamily), *Orthophytum ophiuroides* Louzada & Wand. (Bromelioideae), *Pitcairnia albiflos* Herb. (Pitcairnioideae), *Vriesea gigantea* (Gaud.) (Tillandsioideae) and *V. simplex* (Vell.) Beer (Tillandsioideae) in two species of the genus *Cryptanthus* (*C. burle-marxii* and *C. zonatus*).

Materials and methods

Sample collection and DNA extraction – We sampled fresh leaves of 38 specimens distributed in the Atlantic Forest of northeastern Brazil (states of Pernambuco and Rio Grande do Norte) (Table 1). Additionally, we stored the samples in a sodium chloride-saturated aqueous solution of 2% cetyltrimethylammonium bromide (CTAB) until DNA extraction, as described by Rogstad (1992). We collected specimens of *C. burle-marxii* (one population of 20 individuals and four specimens from selected collections in different locations) and *C. zonatus* (one population of 13 individuals and one specimen from other locality) (Table 1). We identified the specimens following the criteria of Leme and Siqueira Filho (2006). Genomic DNA was extracted from leaves following the protocol of Doyle and Doyle (1987), with modifications described by Weising et al. (2005).

Cross-amplification tests of nuclear microsatellite markers – We tested in *C. burle-marxii* and *C. zonatus* a total of 38 nuclear microsatellite markers previously developed for different Bromeliaceae species, belonging to three subfamilies (Table 2). The 38 loci tested were originally developed by Aoki-Gonçalves et al. (2014), Goetze et al. (2013), Paggi et al. (2008), Palma-Silva et al. (2007) and Neri et al. (2015).

We performed the initial cross-amplification tests using seven specimens, one individual of each population (two of *C. zonatus* and five of *C. burle-marxii*) (Table 1). The loci were amplified by PCR using a Veriti 96-Well Thermal Cycler (Applied Biosystems) in 12-μL reactions containing: ~5 ng DNA template, 5 × GoTaq Master Mix (Promega Corporation), 5 ρmol forward primer, 10 ρmol reverse primer, 1 ρmol universal M13 primer tagged with fluorochromes (NED, FAM, VIC or PET). The forward primers contained a M13 tail with 19 base-pairs (5′-CACGACGTTGTAAAACGAC-3′) at the 5′ end to permit labeling with a fluorescent M13 primer during PCR amplification and genotyping.

We used the touchdown cycling program for all loci as described in detail by Palma-Silva et al. (2007). For three loci Op34, Op82 and Op87 in addition to the touchdown cycling program, we also used the standard cycling program as described in Palma-Silva et al. (2007), with the modification of an annealing temperature of 56 °C. The PCR products were checked on a 1% agarose gel stained with GelRed (Biotum, Hayward, California, USA). We used the 100 bp DNA ladder (Promega) as a molecular size marker. We considered the loci successfully amplified when at least one band of the expected size was observed.

The loci that successfully amplified were genotyped using the 3500 DNA Analyzer automated sequencer (Applied Biosystems) with a standard size GeneScan 500 LIZ (Applied Biosystems). We used the GeneMaker software, version 4.1 (Applied Biosystems) to determine the size range of alleles.

Based on the initial test of the seven individuals representing all of the populations, we choose the polymorphic loci with best pattern of genotyping to be amplified and genotyped in 33 individuals (20 of *C. burle-marxii* from a single population in Parque Estadual das Dunas de Natal, Voucher = *D. Cavalcanti* 757, UFP herbarium; and 13 of *C. zonatus* from a population in the RPPN Serra do Contente, Voucher = *D. Cavalcanti 728*, UFP herbarium), following the protocols described above.

Table 1 Location of the sampled populations and number of individuals collected of *Cryptanthus burle-marxii* and *Cryptanthus zonatus*

Species	State	Municipality	Location	Latitude S	Longitude W	*N*
C. burle-marxii	RN	Natal	Parque Estadual das Dunas de Natal	05°51′39″	35°11′05″	20
C. burle-marxii	RN	Baía Formosa	RPPN Mata Estrela	06°22′40″	35°01′22″	1
C. burle-marxii	PE	Paulista	Estação Ecológica de Caetés	07°55′15″	34°55′15″	1
C. burle-marxii	PE	Ipojuca	Mata do Cupe	08°26′58″	34°59′33″	1
C. burle-marxii	PE	Ipojuca	RPPN Nossa Senhora do Outeiro de Maracaípe	08°31′48″	35°01′05″	1
C. zonatus	PE	Igarassu	Usina São José	07°50′18″	34°59′57″	1
C. zonatus	PE	Gravatá	RPPN Serra do Contente	08°13′48″	35°35′10″	13

Rio Grande do Norte (RN), Pernambuco (PE)

Data analysis

We used the Micro-Checker program version 2.2.3 (Van Oosterhout et al. 2004) to detect the presence of null alleles per locus in the two species of *Cryptanthus*. The genetic diversity per locus of the two species was described by calculating: allelic richness (*AR*), number of alleles (*A*), observed (H_O) and expected (H_E) heterozygosity, and inbreeding coefficient (F_{IS}). These calculations were done using the MSA program version 4.05 (Dieringer and Schlötterer 2003). We tested the Hardy–Weinberg equilibrium (HWE) per locus and per populations using GENEPOP, version 3.5 (Raymond and Rousset 1995).

Results and discussion

From the 38 loci tested, 24 presented successful amplification and were subsequently genotyped (Table 2). Of these, 13 were polymorphic (Table 2), and the 10 loci with best genotyping pattern were chosen for population analysis (Table 3). In the population of *Cryptanthus burle-marxii*, the number of alleles ranged from two to seven per locus and the allelic richness varied from 1.650 to 6.126 per locus (Table 3). The observed and expected heterozygosity ranged from 0.050 to 0.850, and 0.050 to 0.770 per locus, respectively. In the *C. zonatus* population, the number of alleles and the allelic richness varied from two to five per locus (Table 3). The observed and expected heterozygosity ranged from 0.167 to 0.846, and 0.290 to 0.692 per locus, respectively (Table 3). Therefore, the estimated values for number of alleles, allelic richness and heterozygosity per locus did not have large amplitude between the two populations of the species.

The inbreeding coefficient per locus in *C. burle-marxii* varied from −0.198 to 0.240, while in the *C. zonatus* varied from −0.256 to 0.483. The *O. ophiuroides* locus Op52 for *C. zonatus* and *P. albiflos* locus PaC05 for the two species showed significant departure from HWE (Table 3). The presence of null alleles was detected only for *P. albiflos* locus PaC05 in *C. zonatus*. The PaC05 significant departure from HWE in *C. zonatus* may have been due to the possible presence of null alleles. *C. burle-marxii* ($F_{IS} = -0.031$; P value = 0.840) and *C. zonatus* ($F_{IS} = 0.127$; P value = 0.075) were shown to be in Hardy–Weinberg equilibrium (HWE).

Comparing the number of alleles observed in each locus in *Cryptanthus* species with the species for which the loci were previously developed (Goetze et al. 2013 [*A. caudata*]; Aoki-Gonçalves et al. 2014 [*O. ophiuroides*]; Paggi et al. [*P. albiflos*]; and Palma-Silva et al. 2007 [*V. gigantea*]), it can be seen that the majority of the loci (Ac01, Op30, Op69, Op77A, Op78, VgA04) in *Cryptanthus* showed less alleles than those reported by the authors who had developed the loci. Only the *P. albiflos* locus PaC05 presented higher number of alleles (*C. burle-marxii*: 7 alleles and *C. zonatus*: 5 alleles) than the number reported (*P. albiflos:* 3 alleles) by Paggi et al. (2008).

The genetic diversity based on expected heterozygosity (H_E) in the two species (*C. burle-marxii* and *C. zonatus*) was considered low to moderate compared with other bromeliad species which occur in forested areas, such as *V. gigantea* (Palma-Silva et al. 2009) and *Bromelia antiacantha* Bertoloni (Zanella et al. 2011). And it was considered more similar to that found in some bromeliads which occur on rocky outcrops, such as *Alcantarea regina* (Vell.) Harms (Barbará et al. 2008) and *Pitcairnia staminea* Lodd. (Palma-Silva et al. 2011). However, the genetic diversity in the two *Cryptanthus* was considered higher than found in other species which occur on rocky outcrops, such as *Alcantarea geniculata* (Wawra) J.R. Grant (Barbará et al. 2007), *Alcantarea imperialis* (Carriere) Harms (Barbará et al. 2007), *Alcantarea glaziouana* (Leme) J.R. Grant (Barbará et al. 2008) and *P. albiflos* Herb. (Palma-Silva et al. 2011).

The cross-amplification was moderate in both *Cryptanthus* species, about 64% of the loci tested were amplified, and about 35% of the loci tested were polymorphic. The cross-amplification in the three subfamilies tested confirms that the loci can be transferred between the different

Table 2 Cross-amplification of 38 nuclear microsatellite markers previously developed for different Bromeliaceae species in *Cryptanthus burle-marxii* and *Cryptanthus zonatus*

Locus	Species	Subfamily	Amplification	Polymorphic
Ac01	*Aechmea caudata*	Bromelioideae	+	+
Ac11	*Aechmea caudata*	Bromelioideae	+	−
Ac25	*Aechmea caudata*	Bromelioideae	+	−
Ac55	*Aechmea caudata*	Bromelioideae	−	
Ac78	*Aechmea caudata*	Bromelioideae	+	−
Op08	*Orthophytum ophiuroides*	Bromelioideae	−	
Op13	*Orthophytum ophiuroides*	Bromelioideae	+	−
Op17	*Orthophytum ophiuroides*	Bromelioideae	+	+
Op18	*Orthophytum ophiuroides*	Bromelioideae	−	
Op25	*Orthophytum ophiuroides*	Bromelioideae	+	+
Op28	*Orthophytum ophiuroides*	Bromelioideae	−	
Op30	*Orthophytum ophiuroides*	Bromelioideae	+	+
Op34*	*Orthophytum ophiuroides*	Bromelioideae	+	−[a]
Op45	*Orthophytum ophiuroides*	Bromelioideae	−	
Op49	*Orthophytum ophiuroides*	Bromelioideae	−	
Op52	*Orthophytum ophiuroides*	Bromelioideae	+	+
Op53	*Orthophytum ophiuroides*	Bromelioideae	−	
Op63	*Orthophytum ophiuroides*	Bromelioideae	−	
Op69	*Orthophytum ophiuroides*	Bromelioideae	+	+
Op73	*Orthophytum ophiuroides*	Bromelioideae	−	
Op77A	*Orthophytum ophiuroides*	Bromelioideae	+	+
Op77B	*Orthophytum ophiuroides*	Bromelioideae	−	
Op78	*Orthophytum ophiuroides*	Bromelioideae	+	+
Op82*	*Orthophytum ophiuroides*	Bromelioideae	+	−[a]
Op87*	*Orthophytum ophiuroides*	Bromelioideae	+	+[b]
Op92	*Orthophytum ophiuroides*	Bromelioideae	+	+
Op93	*Orthophytum ophiuroides*	Bromelioideae	+	−[a]
Op95	*Orthophytum ophiuroides*	Bromelioideae	+	−[a]
PaC05	*Pitcairnia albiflos*	Pitcairnioideae	+	+
PaD07	*Pitcairnia albiflos*	Pitcairnioideae	+	−[a]
PaZ01	*Pitcairnia albiflos*	Pitcairnioideae	−	
VgA04	*Vriesea gigantea*	Tillandsioideae	+	+
VgC01	*Vriesea gigantea*	Tillandsioideae	+	+
VgF02	*Vriesea gigantea*	Tillandsioideae	−	
Vs1	*Vriesea simplex*	Tillandsioideae	+	−[a]
Vs8	*Vriesea simplex*	Tillandsioideae	+	−[a]
Vs9	*Vriesea simplex*	Tillandsioideae	−	
Vs10	*Vriesea simplex*	Tillandsioideae	−	

[a] Pattern of genotyping unanalyzable

[b] Pattern of genotyping unanalyzable with touchdown cycling program and polymorphic with standard cycling program

* Touchdown cycling program and standard cycling program were used;

species from different subfamilies of Bromeliaceae (Palma-Silva et al. 2007; Paggi et al. 2008; Krapp et al. 2012; Zanella et al. 2012; Goetze et al. 2013; Lavor et al. 2013; Aoki-Gonçalves et al. 2014; Neri et al. 2015).

The ten polymorphic loci transferred to *Cryptanthus burle-marxii*, and *C. zonatus* will be used to assess the genetic diversity and structure of natural populations of these species. Such studies are expected to give insights

Table 3 Genetic diversity parameters estimated for ten polymorphic nuclear microsatellite loci transferred to *Cryptanthus burle-marxii* and *Cryptanthus zonatus*

Locus	*Cryptanthus burle-marxii* ($n = 20$)						*Cryptanthus zonatus* ($n = 13$)					
	Size range (bp)	*A*	*AR*	H_O	H_E	F_{IS}	Size range (bp)	*A*	*AR*	H_O	H_E	F_{IS}
Ac01	283–285	2	1.883	0.100	0.097	−0.040	285–287	2	2	0.385	0.409	0.042
Op17	173–177	3	2.993	0.450	0.476	0.042	173–175	2	2	0.167	0.290	0.418
Op25	171–175	3	2.993	0.450	0.476	0.042	171–173	2	2	0.167	0.290	0.418
Op30	132–138	3	3	0.778	0.660	−0.198	132–140	3	3	0.846	0.692	−0.253
Op52	265–267	2	1.836	0.200	0.185	−0.099	245–273	3	3	0.714	0.648	−0.150*
Op69	114–134	7	5.449	0.850	0.728	−0.185	122–132	4	4	0.818	0.671	−0.256
Op77A	159–161	2	1.650	0.050	0.050	−0.013	159–161	2	2	0.462	0.443	−0.064
Op78	221–233	5	4.960	0.750	0.756	−0.004	227–233	3	3	0.462	0.588	0.202
PaC05	185–207	7	6.126	0.579	0.770	0.240*	165–195	5	5	0.333	0.652	0.483*
VgA04	175–177	2	1.989	0.200	0.185	−0.099	175–177	2	2	0.231	0.409	0.430

Number of alleles (*A*), allelic richness (*AR*), observed heterozygosity (H_O), expected heterozygosity (H_E), inbreeding coefficient (F_{IS})

* Significant departure from HWE ($P < 0.05$)

into the evolutionary history and processes involved in their divergence. With the data genetic structure, we will analyze if it is occurring interspecific gene flow between *C. burle-marxii* and *C. zonatus* and test the hypothesis that they are, in fact, the same species.

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