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Transferability and characterization of microsatellite markers in five Bromeliaceae species belonging to the subfamilies Pitcairnoideae and Bromelioideae

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Abstract: Microsatellite markers previously developed for *Pitcairnia albiflos* Herb. and *Pitcairnia geysksii* L.B.Sm. were used in cross-amplification tests of five other Bromeliaceae species. Ten (76.9%) out of the 13 evaluated pair of primers had positive results for some of the species tested. The number of polymorphic alleles ranged between two and four in most species. H_o values ranged between zero, in *Pitcairnia flammea* Lindl. (PaA05), *Aechmea ramosa* Mart ex Schult *&* Schult and *Billbergia horrida* Regel (PaC05), and one in *Billbergia euphemiae* E. Morren (PaA05, PaA10, PaC05 and PaD07). This study showed that microsatellite markers developed for *P. albiflos* and *P. geysksii* effectively amplified the DNA samples of *Pitcairnia flammea*, *Aechmea nudicaulis*(L.) Griseb., *Aechmea ramosa*, *Billbergia horrida* and *Billbergia euphemiae*, validating the transferability of these markers to species of the *Pitcairnioideae* and *Bromelioideae* subfamilies.
Keywords: *cross-amplification, SSR, genetic diversity, bromeliads, conservation.*

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Resumo: Marcadores microssatélites originalmente desenvolvidos para *Pitcairnia albiflos* Herb. e *Pitcairnia geysksii* L.B.Sm. foram testados para amplificação heteróloga do genoma de cinco outras espécies de Bromeliacea. Dos treze pares de *primers* avaliados, dez (76,9%) geraram resultados positivos para algumas das espécies em estudo. O número de alelos polimórficos por loco variou entre dois e quatro para a maioria das espécies. Os valores de H_O variaram entre zero em *Pitcairnia flammea* Lindl. (PaA05), *Aechmea ramosa* Mart ex Schult *&* Schult e *Billbergia horrida* Regel (PaC05) e um para *Billbergia euphemiae* E. Morren (PaA05, PaA10, PaC05 e PaD07). Este estudo demonstrou que marcadores microssatélites desenvolvidos para *P. albiflos* e *P. geysksii* mostraram-se eficientes para amplificação heteróloga de amostras de DNA de *Pitcairnia flammea, Aechmea nudicaulis*(L.)Griseb.*, Aechmea ramosa, Billbergia horrida e Billbergia euphemiae*, validando a transferibilidade destes marcadores para espécies das subfamílias Pitcairnioideae e Bromelioideae.
Palavras-chave: *amplificação heteróloga, SSR, diversidade genética, bromélias, conservação.*

Introduction

The family Bromeliaceae plays an important role in the conservation of neotropical plant diversity. These epiphytic plants of the Atlantic Forest are among the most visited by birds, also serving as shelter and food for other species (Pizo 1994, Siqueira Filho & Leme 2000). The family Bromeliaceae is organized into three subfamilies: Pitcairnioideae, Tillandsioideae and Bromelioideae (Cronquist 1988), including 3172 species grouped into 58 genera (Luther 2008).

Many members of this family have economic value, e.g. the pineapple plant (*Ananas comosus* (L.) Murril), the 'caroa' (*Neoglaziovia variegata* (Arruda) Mez), which produces fibers and the ornamental plants, especially those of the *Aechmea*, *Billbergia, Canistrum* and *Cryptanthus* genera (Benzig 2000).

Despite their ecological and economic importance, little is known about genetic structure and diversity in natural populations of Bromeliads. According to Cavallari et al. (2006), preservation of the genetic diversity has become the main focus of most conservation programs and knowing the distribution of this diversity within and between natural populations is the first step. Understanding genetic variation within the populations of a given species is an essential prerequisite for conservation action (Barbará et al. 2007), which is essential for the establishment of sustainable forms of economic exploitation.

Therefore, the use of molecular genetic markers to assess genetic diversity of populations plant species occurring in Atlantic Forest has been reported in several studies (Chen et al. 2002, Boneth et al. 2003, Cavallari et al. 2006). Because of their greater convenience, the molecular markers most commonly used in analysis of genetic diversity in plants are polymerase chain reaction-based assays (PCR), particularly microsatellite or Simple Sequence Repeats (SSR) (Litt & Luty 1989), for they are a group of markers highly informative due to their multiallelic and codominant nature, reproducibility, heritability, relative abundance and extensive genome coverage (Powell et al. 1996, Yamamoto et al. 2002).

However, the main limitation on the use of these markers for analysis of genetic diversity in different species is the high cost for developing specific primers. An alternative approach would be the cross amplification of primers. SSR markers can be transferred between related species and genera, which considerably reduces their costs (Ferreira & Grattaplaglia 1998). SSR transferability has been reported to many families of plants, including Fabaceaea (Peakall et al. 1998, Kölliker et al. 2001) Cucurbitaceae (Katzir et al. 1996), Poaceae (Saghai Maroof et al. 1994, Röder et al. 1995, Thiel et al. 2003), Solanaceae (Provan et al. 1996, Smulders et al. 1997, Nagy et al. 2007, Moon et al. 2008); Euphorbiaceae (Yu et al. 2011) and Bromeliaceae (Sarthou et al. 2003, Barbará et al. 2007, Paggi et al. 2008). Thus, in this paper eight microsatellite markers previously developed for *Pitcairnia albiflos* and five markers developed for *Pitcairnia geysksii* were used in cross-amplification tests of five other neotropical Bromeliaceae species (*Pitcairnia flammea*, *Aechmea nudicaulis*, *Aechmea ramosa*, *Billbergia horrida* and *Billbergia euphemiae*).

Materials and Methods

Five markers of microsatellite loci previously developed for *Pitcairnia geysksii* (Sarthou et al. 2003) and eight to *Pitcairnia albiflos* (Paggi et al. 2008) (Table 1) were used in cross- amplification tests of five other Bromeliaceae species belonging to the Pitcairnioideae (*Pitcairnia flammea*) and Bromelioideae (*Aechmea nudicaulis*, *Aechmea ramosa*, *Billbergia horrida* and *Billbergia euphemiae*) subfamilies. A total of 12 *P. flammea* individuals (20° 40' 52.39" S and 41° 20' 43.38" W), eight *A. nudicaulis* (20° 40' 30.83" S and 41° 20' 57.01" W), 12 of *A. ramosa* (20° 40' 30.83" S and 41° 20' 57.01" W), 10 of *B. horrida* (20° 40' 20.1" S and 41° 22' 35.8" W) and four *B. euphemiae* (20° 40' 24,4" S and 41° 20' 55.2" W) were used in the analysis. One voucher for each species (accession nº 5569, nº 55681, nº55657, nº 55664 and nº 55660 respectively) was deposited in the CESJ Herbarium at Universidade Federal de Juiz de Fora (UFJF).

Leaf samples were collected from individuals of these species in natural populations occurring in fragments of the Atlantic Forests, in Burarama, Cachoeiro de Itapemirim, ES. DNA of plant samples were extracted and purified using the cetyltrimethylammonium bromide (CTAB) extraction method, as described by Doyle & Doyle (1990).

In order to improve the PCR result, the optimal annealing temperatures of each pair of primers to be tested were determined (between 48 and 56 °C). In all cases, microsatellite loci were amplified in a 15 µL volume containing 0.4 µM of each primer, 1 U Taq DNA polymerase, 0.1 mM of each dNTP, 1 × $MgCl_2$ – free reaction buffer (10 mM Tris-HCl pH 8.3 and 50 mM KCl), 2 mM $MgCl_2$ and 30 ng of template DNA.

Amplifications were performed using a Techne TC-412 thermal cycler under the following conditions: 5 minutes denaturation at 94 °C followed by 30 cycles of 1 minute of initial denaturation at 94 °C, 1 minute of annealing temperature at 54 °C and 1 minute of extension at 72 °C, and elongation at 72 °C for 7 minutes.

Amplified fragments were separated by electrophoresis on 2.5% agarose gel containing 0.02 ug/mL ethidium bromide, 1x TBE buffer (0.89 M Tris-HCl pH 8.3, 0.89 M boric acid and 0.02 M EDTA), at 110 volts for approximately three hours. Afterwards, the gels were photographed under UV light, using the gel documentation system Biolocus L PIX (Loccus Biotecnologia®). Gel electrophoresis was used to assess the number and size of amplified fragments, as well as polymorphism detection.

Analyses of genetic variability at the microsatellite *loci* were done using the genotypes obtained for all five species of bromeliads evaluated in this study. The number of alleles per locus and the observed and expected heterozygosities under Hardy-Weinberg equilibrium were estimated. These analyses and the test for deviation from Hardy-Weinberg expectations were performed with Genes program (Cruz 2008).

Results and Discussion

Ten of the 13 microsatellite loci evaluated generated amplification products. In PaA10, PaB12, PaC05, PaD07, PaZ01 and Pit5 loci cross-amplification was successful for the five study species, for the annealing temperature of these primers was identical (54 °C). The other markers had positive results for some of these species (Table 2). In *P. flammea,* there was positive amplification to 10 (76.92%) microsatellites loci, of which only three were monomorphic, in *A. Nudicaulis,* six (46.15%) markers generated amplification products, since in five of them polymorphism was detected, to *A. ramosa* and *B. horrida,* eight (61.53%) markers were successfully amplified, with only one monomorphic locus and, in *B. euphemiae,* nine (69.23%) markers generated positive results, as in seven polymorphism was detected.

Paggi et al. (2008) used the same markers previously developed for *P. albiflos* (subfamily Pitcairnioideae) to test cross amplification in 16 other bromeliad species, six of which belong to subfamily Pitcairnioideae, eight to family Bromelioideae and two to family Tillandsioideae. In this paper, PaB12, PaC05 and PaD07 markers showed positive results of amplification in all species belonging to subfamily Pitcairnioideae. PaA05, PaA09 and PaB11 markers exhibited exclusive transference only to this subfamily. On the other hand, PaA10, PaC05, PaD07 and PaZ01 markers were successfully transferred to species distributed in the three subfamilies.

Table 1. Characteristics of microsatellite loci used on heterologous amplification tests, including the name of the species for which the marker was previously developed, locus name, primer sequences, microsatellite sequence, size of fragments detected in the original citation and GenBank accession number.

Tabela 1. Características dos loci microssatélites usados nos testes de amplificação heteróloga, incluindo o nome da espécie para a qual o marcador foi originalmente desenvolvido, o nome do *locus*, a seqüência do *primer*, a seqüência do microssatélite, o tamanho do fragmento detectado na citação original e o número de acesso no GeneBank.

Species name	Locus	Primer sequences (5'-3')	SSR motif	Size range (bp)	Accession number
Pitcairnia albiflos	PaA05[a]	F: ACCGGGTTCAGGGAAAATAC R: TTGAGGCTAAGAGCGAGGAG	$(TTC)_{10}NN(CT)_{17}$	228-258	EU293085
Pitcairnia albiflos	PaA09[a]	F: AGAAGAGAACCCACCCCAAG R: GTGTTCCGCGACACTACAAA	$(CT)_{25}$	191-213	EU293086
Pitcairnia albiflos	PaA10[a]	F: AACCATTGACATCCGCTGTT R: CTTCGGAAGCTCCTCTGGAT	$(ATG)_{10}$	146-149	EU293087
Pitcairnia albiflos	PaB11[a]	F: AGAGGCTGAGAGAGGTAAACCA R: CGAGCCCTCTTTCTGAACC	$(AG)_{9}$	159-171	EU293088
Pitcairnia albiflos	PaB12[a]	F: CCCGAGGGACATTCTCTCTT R: CATGGCGCAGTAGTGTTTTC	$(CT)_{19}NN(CT)_{4}NN(CT)_{4}NN(TG)_{7}(TC)_{5}$	219-259	EU293089
Pitcairnia albiflos	PaC05[a]	F: TCGATGTCGACGGTAGTGAG R: TCCTCTCGCTTTGATTCACC	$(AG)_{18}NN(GA)_{7}$	149-153	EU293090
Pitcairnia albiflos	PaD07[a]	F: TCCATGTGCCTCATCATAGC R: TGCCCACAAAGCATATCAGT	$(TG)_{10}$	233-239	EU293091
Pitcairnia albiflos	PaZ01[a]	F: TGACCAGATAGCACCATCCA R: TTGAGTGTTGGAGCCCACTT	$(AG)_{20}$	185-199	EU293092
Pitcairnia geyskesii	Pit2[b]	L: TTAGCGGCAGTTAGAAACAGG R: GATCTCCGATGTCTTGTTAGG	$(CT)_{13}$	191-219	AY188957
Pitcairnia geyskesii	Pit4[b]	L: CCGACTCTATCGTCAAAGG R: TTATCACCTCCCATGTCTCC	$(CT)_{16}$	214-230	AY188958
Pitcairnia geyskesii	Pit5[b]	L: TTGAGCCATGAACAATAGGG R: AGAATTCTAGTGGCAGTCCTC	$(GA)_{20}$	310-343	AY188959
Pitcairnia geyskesii	Pit6[b]	L: AAAGCTACATCGTCGAAAACAAC R: CAATCAAGTTTCGGGTCACTAC	$(CT)_{11}$	108-120	AY188960
Pitcairnia geyskesii	Pit9[b]	L: AACCATTACATGCACCCTCAC R: TCACTGGGGAAGCCATAGAG	$(TC)_{13}(AT)_{9}$	113-129	AY188962

[a]markers described by Paggi et al. (2008) and [b]markers described by Sarthou et al. (2003).

In the present study, microsatellite markers, which until then had been described only to species of the genus *Pitcairnia*, such as PaA05, PaA09 and PaB11, were found to effectively generate cross amplification in species of *Aechmea* and *Billbergia* (Table 2), validating the transferability of these markers in these genus. Similar results were found by Palma-Silva et al. (2007) who successfully performed the transposition of SSR markers developed from species of subfamily Tillandsioideae (*Vrissea gigantea* and *Alcantarea imperialis*) to Bromelioideae and Pitcairnioideae species.

Another important aspect of our findings was the polymorphic markers identified for each one of the assessed species. Considering only the polymorphic loci, the number of alleles per locus ranged between two and four for most of the species (Table 2). These findings differ from those obtained by Sarthou et al. (2003) and Paggi et al. (2008), regarding the number of alleles detected and the size of fragments generated. According to Wang et al. (2009), this may have occurred because of the different methodologies used, namely capillary electrophoresis in the genotyping studies where these markers were originally described and agarose gel electrophoresis, in the present study. Another possible explanation is that these differences are the result of variations in the number of tandem repeat polymorphisms on the tested loci. In their evaluation of the percentage of transferability of SSR markers developed from *Nicotiana tabacum* to other species of the same genus, Moon et al. (2008) also found differences in reproducibility of amplifications, with variable fragment sizes, compared to the expected. Bravo et al. (2006) argue that there may be considerably variation both in the number of repetitions as well in levels of polymorphism between the species for which SSR markers were previously developed and the species that showed a cross reaction.

Regarding genotype distribution and values of the expected (H_E) and observed (H_O) heterozygosity (Table 2), in *P. flammea*, these ranged between 0.19 (PaA05) and 0.62 (PaZ01), and from zero (PaA05) to 0.64 (PaC05) respectively. In *A. Nudicaulis*, H_E ranged from 0.33 (PaA10) to 0.64 (PaZ01) and H_O between 0.25 (Pit5) and 0.63 (PaZ01) (Table 2). In *A. ramosa* and *B. horrida*, H_E ranged from 0.37 (PaA10, Pit5) to 0.63 (PaB12, PaZ01) and the values of H_O were detected ranging between zero (PaC05) and 0.77 (PaA05, PaB12) and from 0.25 (Pit5) to 0.77 (PaB12), respectively. To *B. euphemiae*, H_E ranged from 0.22 (PaA09) to 0.59 (PaZ01) and H_O ranged between zero (Pit5) and one (PaA05, PaA10, PaC05 and PaD07). Moreover, significant deviations from Hardy-Weinberg equilibrium were observed in some loci in population samples of the five bromeliad species. Similar results were reported by many authors in cross amplification studies using SSR markers for different species (Sarthou et al. 2003, Gimenes et al. 2007, Paggi et al. 2008, Nazareno et al. 2009). In most cases, this can be attributed to the existence of null alleles (alleles not amplified in some genotypes) or to sampling errors, causing the Wahlund effect. Still according to Wright (1965), the level of heterozygosity found in a population is highly dependent on the mating system and on the evolutionary history of the species, as well as on a number of other factors.

Therefore, given the high success rate in the cross amplification test and the level of polymorphism detected, the microsatellite

Table 2. Results of heterologous amplifications of microsatellite loci in DNA samples of individuals of *Pitcairnia flammea*, *Aechmea nudicaulis*, *Aechmea ramosa*, *Billbergia horrida* and *Billbergia euphemiae* species.

Tabela 2. Resultados das amplificações heterólogas de loci microssatélites em amostras de DNA de indivíduos das espécies *Pitcairnia flammea, Aechmea nudicaulis, Aechmea ramosa, Billbergia horrida e Billbergia euphemiae*.

Specie	Loci	PaA05	PaA09	PaA10	PaB11	PaB12	PaC05	PaD07	PaZ01	Pit5	Pit9	Média
P. flammea (n = 12)	Size range (bp)	185-210	215-270	60	90	240-290	65-85	60-100	250-280	320	70-90	
	Number of alleles	2	3	1	1	3	3	2	3	1	2	
	H_O	0	0.36	-	-	0.4	0.64	0.09	0.2	-	0.54	0.31
	H_E	0.19**	0.43*			0.46	0.53	0.23*	0.62**		0.5	0.42
A. nudicaulis (n = 8)	Size range (bp)	-	-	140-160	-	190	165-195	250-280	160-200	360-400	-	
	Number of alleles	-	-	2	-	1	2	2	4	3	-	
	H_O	-	-	0.43	-	-	0.6	0.5	0.63	0.25	-	0.48
	H_E			0.33*			0.62	0.37*	0.64	0.55**		0.50
A. ramosa (n = 12)	Size range (bp)	380-460	-	130-160	60-110	80-120	85-95	230-260	160-190	380	-	
	Number of alleles	2	-	2	2	4	2	2	3	1	-	
	H_O	0.77	-	0.16	0.57	0.77	0	0.75	0.25	-	-	0.47
	H_E	0.47*		0.37	0.41*	0.63**	0.42**	0.46*	0.63*			0.48
B. horrida (n = 10)	Size range (bp)	280	-	130-160	140-160	240-255	250-270	190-210	170-200	345-365	-	
	Number of alleles	1	-	2	2	2	2	2	3	2	-	
	H_O	-	-	0.55	0.57	0.77	0.44	0.63	0.66	0.25	-	0.55
	H_E			0.5*	0.48	0.5*	0.34**	0.43**	0.62*	0.37*		0.46
B. euphemiae (n = 4)	Size range (bp)	390-420	140-160	180-210	-	180	190-210	230-260	175-195	320-340	380	
	Number of alleles	2	2	2	-	1	2	2	3	2	1	
	H_O	1	0.25	1	-	-	1	1	0.5	0	-	0.67
	H_E	0.5*	0.22	0.5*			0.5*	0.5*	0.59	0.5*		0.47

The annealing temperature was the same for all the markers - 54 °C; statistically significant deviation from Hardy-Weinberg equilibrium (*P < 0.005; **P < 0.001).

markers used here can make a significant contribution to research on genetic structure of natural populations of Bromeliads. As described by Barbará et al. (2007), the findings of the present study provide subsidies to the establishment of effective strategies for conservation of forest genetic resources. Another important aspect is highlighted by Noor & Feder (2006). According to these authors, the possibility of cross amplification of genetic markers allow comparative studies among closely related taxa, as well as understanding of the genetic mechanisms of speciation and population divergence.

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