



Genetic diversity of Bromeliaceae species from the Atlantic Forest

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ABSTRACT. The Bromeliaceae family includes a range of species used for many purposes, including ornamental use and use as food, medicine, feed, and fiber. The state of Espírito Santo, Brazil is a center of diversity for this family in the Atlantic Forest. We evaluated the genetic diversity of five populations of the Bromeliaceae family, including specimens of the genera *Aechmea*, *Billbergia* (subfamily Bromelioideae), and *Pitcairnia* (subfamily Pitcairnioidea), all found in the Atlantic Forest and distributed in the state of Espírito Santo. The number of alleles per locus in populations ranged from two to six and the fixation

index (F), estimated for some simple sequence repeats in bromeliad populations, was less than zero in all populations. All markers in the *Pitcairnia flammea* population were in Hardy-Weinberg equilibrium ($P < 0.05$). Moreover, significant deviations from Hardy-Weinberg equilibrium were observed at some loci in populations of the five bromeliad species. In most cases, this can be attributed to the presence of inbreeding or the Wahlund effect. The genetic diversity indices of five species showed greater allelic richness in *P. flammea* (3.55). Therefore, we provide useful information for the characterization of genetic diversity in natural populations of *Aechmea ramosa*, *Aechmea nudicaulis*, *Billbergia horrida*, *Billbergia euphemia*, and *P. flammea* in Atlantic Forest remnants in the south of Espírito Santo state.

Key words: Microsatellites; Bromeliads; Genetic diversity

INTRODUCTION

The Bromeliaceae family includes approximately 60 genera and 3170 species (Luther, 2008; Givnish et al., 2011). The only exception to the uniquely American pattern of distribution is the *Pitcairnia feliciana* species (A. Chev.) Harms & Mildbr, which is located in the region of Guinea, along the West Africa coast (Benzing, 2000). The Bromeliaceae family is one of the most morphologically and ecologically diverse flowering plant families native to the tropics and subtropics of the New World (Givnish et al., 2011).

Traditionally, this family has been divided into three subfamilies: Bromelioideae, Pitcairnioideae, and Tillandsioideae, based on Cronquist (1988). However, Givnish et al. (2011) confirmed a phylogeny based on eight plastid regions, with the new classification including eight subfamilies: (*Brochinioideae*, *Lindmanioideae*, *Tillandsioideae*, *Hechtioideae*, *Navioideae*, *Pitcairnioideae*, *Puyoideae*, and *Bromelioideae*) according to the morphological characteristics of flowers, fruits, and seeds, and on molecular data (Givnish et al., 2007; Givnish et al., 2011).

Plants in the Bromeliaceae family provide excellent models for the study of genetic diversity (Zanella et al., 2012; Hmeljevski et al., 2013), population genetics (Lavor et al., 2014), as well as the divergence between populations and species (Sarhou et al., 2001; Barbará et al., 2007; Palma-Silva et al., 2009). Bromeliads are an important and characteristic element of Neotropical forests, especially in the Brazilian Atlantic Forest (BAF), where they are among those families with the greatest richness and diversity (Martinelli et al., 2008).

Nevertheless, relatively few genetic studies have examined Neotropical plant species and investigated genetic aspects of BAF bromeliads by comparing temperate forests (Cavallari et al., 2006; Barbará et al., 2007; Palma-Silva et al., 2009; Versieux et al., 2012; Zanella et al., 2012; Lavor et al., 2014; Goetze et al., 2015). According to Zanella et al. (2012), only 20 species of the following nine genera have been previously evaluated, and most of the studied species are endemic to the Atlantic rainforest in southeastern Brazil: *Aechmea*, *Alcantarea*, *Bromelia*, *Dyckia*, *Encholirium*, *Pitcairnia*, *Puya*, *Tillandsia*, and *Vriesea*.

However, this biome has been severely destroyed and reduced to only 13% approximately of its original area, which is the main threat to BAF flora and fauna (Ribeiro, 2009). Despite this fragmentation, Martinelli et al. (2008), identified the state of Espírito Santo

as the second largest region for species richness and endemism of this family in the Atlantic Forest; however, it is considered one of the families with the highest number of threatened taxa in the state (Kollmann et al., 2007).

According to Cavallari et al. (2006), the conservation of genetic diversity has become the goal of many conservation programs, and knowledge of the distribution of such diversity within and among natural populations is the first step in this process. Therefore, research on population genetic diversity is essential to provide information on the risk of species extinction and to design effective conservation strategies (Frankham et al., 2002). In this context, molecular markers are widely used for studying genetic variability (Cavallari et al., 2006; Barbará et al., 2007, 2009; Lavor et al., 2014; Goetze et al., 2015), and several markers have been developed for Bromeliaceae: *Pitcairnia* (Sarhou et al., 2003; Paggi et al., 2008), *Alcantarea* (Palma-Silva et al., 2007), *Dyckia* (Zanella et al., 2012), *Encholirium* (Hmeljevski et al., 2013), *Orthophytum* (Aoki-Gonçalves et al., 2014), and recently, *Vriesea* (Neri et al., 2015).

Here, we evaluate the genetic structure of five populations of the Bromeliaceae family, including specimens of the genera *Aechmea*, *Billbergia* (subfamily Bromelioideae), and *Pitcairnia* (subfamily Pitcairnioideae). In addition, we identified and quantified genetic variability using simple sequence repeat (SSR) markers previously validated by Miranda et al. (2012).

MATERIAL AND METHODS

Study site and sample collection

The collection sites were located in the priority ecological corridor Burarama - Pacotuba - Cafundó, established in 2004 by the Project Ecological Corridors of the Institute for the Environment of the Espírito Santo state (IEMA). The purpose of this project is to connect the private reserve of the natural patrimony Cafundó Farm to the Pacotuba National Forest, with a view to decrease the effects of fragmentation in the Atlantic Forest (Projeto Corredores Ecológicos, 2006).

Populations of five species were sampled in the locality Burarama I: 32 *Aechmea ramosa* individuals (20°40'30.83"S and 41°20'57.01"W) and 35 *Aechmea nudicaulis* individuals (20°40'30.83"S and 41°20'57.01"W); locality Burarama II: 31 *Billbergia horrida* individuals (20°40'20.1"S and 41°22'35.8"W) and 26 *Billbergia euphemiae* individuals (20°40'24.4"S and 41°20'55.2"W); and finally, locality Burarama III: 40 *Pitcairnia flammea* individuals (20°40'52.39"S and 41°20'43.38"W), all located in Atlantic Forest remnants in southern Espírito Santo State (Brazil). One voucher for each species (accession No. 5569, 55681, 55657, 55664, and 55660) was deposited in the CESJ Herbarium at Universidade Federal de Juiz de Fora (UFJF).

Microsatellite markers

We evaluation of SSR markers for two loci markers originally developed for *Pitcairnia geyskii* by Sarhou et al. (2003) and eight for *Pitcairnia albiflos* (Paggi et al., 2008). These markers were previously evaluated by Miranda et al. (2012) and were found to be heterologous in amplification assays of *A. ramosa*, *A. nudicaulis*, *B. horrida*, *B. euphemiae*, and *P. flammea*.

DNA extraction and amplification conditions

Samples of leaf tissue from each plant were used for DNA extraction and purification

as described by Doyle and Doyle (1990). Polymerase chain reaction (PCR) amplifications were performed in a 15- μ L volume containing 0.4 μ M each primer, 1 U Taq DNA polymerase, 0.1 mM each dNTP, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM MgCl₂, and 30 ng template DNA. Amplifications were performed with a Veriti Thermal Cycler (Applied Biosystems) using a cycling program of 5 min at 94°C, followed by 35 cycles consisting of three steps: a) 1 min at 94°C, b) 1 min at 54°C, c) 1 min at 72°C, with a final step of 7 min at 72°C. Amplified fragments were separated by electrophoresis on 8% acrylamide gel containing 0.02 μ g/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 V for approximately 4 h. At the end of the run, the gels were photographed under ultraviolet light.

Data analysis

The acrylamide gels were analyzed to determine the genetic diversity of each locus, and the population was evaluated using the following descriptive summary statistics: allelic richness (R), number of alleles (A), observed (H_O) and expected (H_E) heterozygosity, fixation index (F), and Hardy-Weinberg equilibrium (HWE) using GenALEX (Peakall and Smouse, 2006). The MStools program (Park, 2001) was used to calculate the polymorphic information content (PIC) within populations.

RESULTS

The values of parameters obtained for SSRs in each of the populations are presented in Table 1.

Table 1. Global microsatellite values for each population and estimates of genetic variability, based on Nei (1973).

Population		PaA05	PaA10	PaB11	PaB12	PaC05	PaD07	PaZ01	Pit05	
<i>Aechmea ramose</i> (N = 32)	\hat{A}	2	2	3	5	2	4	4	3	
	H_O	0.285	0.300	0.500	0.187	0.125	0.781	1.000	0.074	
	H_E	0.249	0.304	0.436	0.670	0.436	0.637	0.654	0.519	
	PIC	0.214	0.254	0.350	0.611	0.337	0.557	0.575	0.435	
	F	-0.167	-0.002	-0.164	0.709	-0.245	-0.554	0.855	0.716	
	HWE	0.378	0.992	0.290	0.523	0.000*	0.000*	0.000*	0.000*	
		PaA10	PaB12	PaC05	PaZ01	Pit05				
<i>Aechmea nudicaulis</i> (N = 35)	\hat{A}	2	3	4	6	4				
	H_O	0.714	0.178	0.323	0.875	0.787				
	H_E	0.480	0.168	0.548	0.808	0.628				
	PIC	0.362	0.156	0.491	0.765	0.554				
	F	-0.507	-0.081	0.401	-0.100	-0.274				
	HWE	0.003*	0.966	0.054	0.000*	0.221				
		PaA05	PaB11	PaB12	PaC05	PaD07	PaZ01	Pit05		
<i>Billbergia horrida</i> (N = 31)	\hat{A}	2	2	4	2	6	4	3		
	H_O	0.300	0.656	0.300	0.100	0.758	0.684	0.677		
	H_E	0.260	0.507	0.637	0.096	0.743	0.689	0.571		
	PIC	0.222	0.374	0.565	0.905	0.693	0.619	0.501		
	F	-0.176	-0.314	0.521	-0.053	-0.040	-0.019	-0.204		
	HWE	0.334	0.076	0.000*	0.776	0.000*	0.040			
		PaA05	PaA09	PaA10	PaB12	PaC05	PaD07	PaZ01	Pit05	Pit09
<i>Billbergia euphemia</i> (N = 26)	\hat{A}	2	2	2	2	2	6	4	4	3
	H_O	0.434	0.476	0.44	0.000	0.238	0.791	0.391	0.391	0.347
	H_E	0.347	0.371	0.392	0.435	0.396	0.826	0.607	0.561	0.501
	PIC	0.282	0.297	0.310	0.335	0.311	0.781	0.550	0.487	0.418
	F	-0.278	-0.313	-0.143	1.000	0.384	0.021	0.342	0.284	0.291
	HWE	0.183	0.152	0.473	0.000*	0.078	0.000*	0.000*	0.001*	0.131
		PaA05	PaA09	PaA10	PaB11	PaB12	PaC05	PaD07	PaZ01	Pit05
<i>Pitcairnia flammea</i> (N = 40)	\hat{A}	4	5	3	2	3	4	4	3	4
	H_O	0.941	0.825	0.025	0.575	0.947	0.645	0.948	0.923	1.000
	H_E	0.586	0.649	0.073	0.414	0.544	0.708	0.554	0.530	0.545
	PIC	0.489	0.572	0.071	0.325	0.429	0.646	0.445	0.410	0.430
	F	-0.628	-0.286	0.657	-0.404	-0.764	0.074	-0.733	-0.764	-0.860
	HWE	0.103	0.234	0.057	0.043	0.072	0.124	0.203	0.178	0.275

*P < 0.05. \hat{A} = number of alleles per locus; H_O = observed heterozygosity; H_E = expected heterozygosity under Hardy-Weinberg; PIC = polymorphic information content; F = fixation index; HWE = Hardy-Weinberg equilibrium; n = number of individuals.

The number of alleles per locus in each population ranged from two to six. The expected and observed heterozygosity ranged from 0.073 (PaA10 in *P. flammea*) to 0.826 (PaD07 in *B. euphemia*), and from 0.000 (PaB12 in *B. euphemia*) to 1.000 (PaZ01 in *A. ramosa* and Pit05 in *P. flammea* population) respectively. The polymorphic information content ranged from 0,071 (PaA10 in *P. flammea* population) to 0.905 (PaC05 in *B. horrida* population), and some markers obtained were highly informative ($PIC > 0.5$). For example, PIC values of 0.765 (PaZ01 in *A. nudicaulis*) and 0.781 (PaD07 in *Billbergia*) were obtained. The fixation index (F), estimated at some SSR in bromeliad populations was less than zero in all populations. SSRs in the *B. horrida* population (except for PaB12) promote inbred escape (outbreeding); the opposite was observed, such that some loci are inbred (e.g., from 0.657 to in the PaA10 *P. flammea* population), or the genotypic frequencies in these alleles within populations are not in Hardy-Weinberg equilibrium.

Regarding the estimation of HWE for each locus, only *P. flammea* populations exhibited markers that were all in HWE ($P < 0.05$). Average intra-population genetic diversity was assessed for each species, and was also demonstrated by estimating allelic richness, heterozygosity, PIC, and fixation index in Table 2.

Table 2. Genetic parameters obtained for each species.

Species	R_s	H_o	H_e	PIC	F
<i>Aechmea ramosa</i>	3.12	0.407	0.481	0.416	0.143
<i>Aechmea nudicaulis</i>	3.16	0.576	0.519	0.465	-0.110
<i>Billbergia horrida</i>	3.00	0.430	0.435	0.554	-0.041
<i>Billbergia euphemia</i>	3.00	0.390	0.483	0.419	0.177
<i>Pitcairnia flammea</i>	3.55	0.759	0.505	0.424	-0.112

R_s = allelic richness; H_o = observed heterozygosity; H_e = expected heterozygosity under Hardy-Weinberg; PIC = polymorphic information content; F = fixation index.

Regarding genetic diversity parameters, allelic richness ranged from 3.55 (*P. flammea*/locality Burarama III) to 3.00 (*B. horrida* and *B. euphemia*/locality Burarama II); expected heterozygosity ranged from 0.435 (*B. horrida*) to 0.519 (*A. nudicaulis*), and the fixation index was between -0.110 (*A. nudicaulis*) and 0.177 (*B. euphemia*). Furthermore, in the present study, the SSRs utilized were efficient at indicating moderate-to-high polymorphism between species, as described by Xie et al. (2010).

DISCUSSION

Overall, the 10 microsatellites used amplified some of the Bromeliad species evaluated in this study. Most of these markers were polymorphic, with the exception of PaD07A and PaA10, which were monomorphic for the populations of *A. nudicaulis* and *B. horrida*, respectively.

The inbreeding populations for *A. nudicaulis* ($F = -0.110$), *B. horrida* ($F = -0.041$), and *P. flammea* ($F = -0.122$) may be considered null, since 95%CI of the estimates was not significant, that is, it includes zero. Considering panmixia and HWE, similar results were obtained in previous studies of population genetic diversity for *A. regina* ($F = -0.051$) (Barbará et al., 2009) and *P. geyskii* ($F = -0,037$) (Sarhou et al., 2001).

Moreover, significant deviations from Hardy-Weinberg equilibrium were observed in some loci from populations belonging to the five bromeliad species (Table 1). In most cases, this can be attributed to the presence of inbreeding or to the Wahlund effect (Hartl and Clark,

1997). In populations with the highest number of loci with deviations (*A. ramosa* and *B. euphemia*), significant values were also found for fixation indexes, giving positive values for intrapopulation inbreeding.

The genetic diversity indices of five species showed there is greater allelic richness in *P. flammea* (3.55). This is due to the larger sample size of the population, with 40 individuals. Furthermore, the sampled area (Burarama III) was difficult to access, with the presence of dense forest, which is in contrast to other sampled locations (Burarama I and II), in which different degrees of human action were observed.

Regarding the overall distribution of genotypes, the observed and expected heterozygosities were similar (Table 2). Unlike the pattern found for *Alcantarea imperialis* (Carrière) JR Grant and *Aechmea geniculata* (Wawra) JR Grant (Barbará et al., 2007), in *Vriesea gigantea* (Palma-Silva et al., 2009), and also *Vriesea minarum* (Lavor et al., 2014), the observed heterozygosity was lower than expected because of a deficit of heterozygotes. These data further suggest that an excess of homozygotes could be due to selfing or biparental inbreeding, since *V. minarum* exhibits a mixed reproductive strategy (outcrossing + self-compatibility), or due to a feature of the subfamily Tillandsioideae. Matallana et al. (2010) suggested that *A. nudicaulis*, *B. horrida*, and *B. euphemia* have self-incompatible (SI) mating systems, which promote allogamy, representing positive and various empirical models that suggest outinbreeding that is evolutionarily advantageous due to selective pressures favoring genetic variability. Moreover, *Pitcairnia flammea* according to Wendt et al. (2002), presents reproduction type of self-compatible or partially self-compatible. Our data support the presence of substantial genetic diversity among populations of *A. ramosa*, *A. nudicaulis*, *B. horrida*, *B. euphemia*, and *P. flammea*.

These findings provide useful information for the characterization of genetic diversity in natural populations of *A. ramosa*, *A. nudicaulis*, *B. horrida*, *B. euphemia*, and *P. flammea* in the Atlantic Forest remnants in the south of Espírito Santo state. According to Bizoux and Mahy (2007), clonal and sexual reproduction, demography, genetic structure within and among populations, gene flow, and mating systems of bromeliads are important for the development of successful conservation strategies. These data on the genetic diversity and distribution of genotypes among populations are essential for determining the conservation status of species and may be used for decision making regarding the delineation of protected areas.

Conflicts of interest

The authors declare no conflict of interest.

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