Genetic diversity of *Vriesea cacuminis* (Bromeliaceae): an endangered and endemic Brazilian species

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ABSTRACT. Data about the genetic structure can help to understand the evolutionary process of natural populations as well as to drive strategies of conservation. *Vriesea cacuminis*, an endemic Brazilian Bromeliad, has been found in 2 areas of Minas Gerais State. One is a legal preservation unit (Ibitipoca State Park) and the other an unprotected area (Serra Negra). The 2 areas belong to the Mantiqueira Mountain Range Complex; both are characterized by steep relief with high altitudes and by heterogenic vegetation formed by a mosaic of rocky fields and forest fragments. According to International Union for Conservation of Nature criteria, *V. cacuminis* is designated as "vulnerable". We examined the genetic variability and population structure of 70 individuals (3 populations) of *V. cacuminis*, using 16 ISSR markers. Although *V. cacuminis* is considered a rare species, the estimated genetic diversity was found to be relatively high (Shannon index = 0.33; percentage

of polymorphic bands = 87%). The populations were found not to be structured (AMOVA test, $\Phi_{ST} = 0.16$), probably due to the cross-breeding. Based on Bayesian analysis, this species includes one cluster containing the populations from Ibitipoca State Park and another cluster including the population from Serra Negra. This information will help determine strategies to maintain the genetic variability of these populations.

Key words: Bromeliaceae; *Vriesea cacuminis*; Endemism; Conservation; Genetic diversity

INTRODUCTION

Genetic structure data on natural plant populations have helped to understand the adaptation process, speciation, gene flow, and how ecological and evolutionary processes can influence the dynamics of natural plant populations (Barbará et al., 2009; Lousada et al., 2011). Moreover, these studies can help to define priority locations to be preserved by law and also to develop conservation strategies (Holsinger and Gottieb, 1991). The amount of genetic variability can also be related to the survival time of one population, since high genetic diversity normally increases the chance of adaptation and consequently influences how long it can survive (Neel and Cummings, 2003). Although it is possible to observe small populations with restricted distribution and high variability (Viccini et al., 2004; Hmeljevski et al., 2011), in general, rare and endemic species show a lower level of genetic diversity compared to widely distributed ones (Gitzendanner and Soltis, 2000).

Due to the occurrence of several species at high altitudes, bromeliads can help us to understand the speciation process and genetic variability distribution in tropical inselbergs. The family Bromeliaceae comprises nearly 3172 species distributed in 58 genera (Luther, 2010) mainly with Neotropical distribution. An exception is *Pitcairnia feliciana*, which occurs in Africa (Jacques-Felix, 2000). Around 1030 of 1207 Brazilian species are endemic, making this country one of the most important ones regarding the family diversity (Forzza et al., 2013). On the other hand, the destruction of the ecosystem and the predatory extraction of those plants have caused a drastic reduction in the number and size of populations and, therefore, loss of genetic diversity (Martinelli et al., 2008; Barbará et al., 2009). *Vriesea cacuminis* L.B.Sm. is an example of a rare bromeliad, which is restricted to 2 places in Minas Gerais State, Brazil, 30 km apart: Ibitipoca State Park, a State conservation unit, and Serra Negra, an unprotected area (Versieux, 2011). Both locations belong to the Mantiqueira Range Complex and are characterized by a steep relief with altitudes ranging from 900 to 1794 m and heterogeneous vegetation composed of a mosaic of rocky fields and forest fragments (Menini et al., 2009). The species can be seen in the rocky landscapes mainly at altitudes higher than 1400 m (Monteiro and Forzza, 2008). Due to its restricted distribution, *V. cacuminis* has been included in the Brazilian lists of threatened species, classified as vulnerable (Versieux and Wendt, 2007; Drummond et al., 2009), and it was categorized as a species with insufficient data by the official Brazilian Red List (MMA, 2008).

Nowadays, genetic variability has been investigated mainly at the DNA level. The inter-simple sequence repeat (ISSR) is one of the markers that has been used to study the variability of natural plant populations. This PCR-based method does not require prior genomic

sequence information since the ISSR primers anneal close to microsatellite regions, which occur randomly in eukaryotic genomes. As a result, several markers can be amplified from a single reaction and can be highly variable at the intraspecific level (Wolfe, 2005).

Here, we report on the use of ISSR markers to investigate the genetic variability of all known populations of *V. cacuminis*. It was possible to compare the diversity between protected and unprotected areas. In addition, possible mechanisms of species dispersion between the 2 mountain ridges and also how population variability can be maintained are discussed.

MATERIAL AND METHODS

Population sampling

Leaves of *V. cacuminis* were collected from 2 populations at Ibitipoca State Park (Peão, S21°42'7.9" and W43°52'35.1", with an elevation of 1578 m and Cruzeiro, S21°41'44.4" and W43°53'49.6", with an elevation of 1670 m) and 1 population in the Serra Negra range (S21°57'57" and W43°53'13.1", with an elevation of 1592 m), both located in Minas Gerais State, Brazil (Figure 1). Nearly 300 mg of young leaves was collected from 20, 29, and 21 individuals of Peão, Cruzeiro, and Serra Negra populations, respectively. Due to wider distribution of Cruzeiro population, the individuals were sampled at 4 different points (Figure 1).
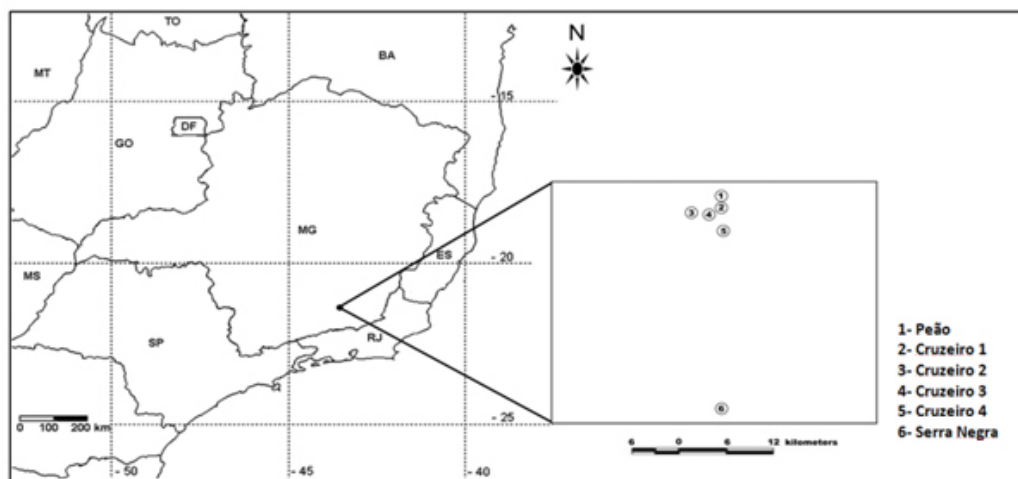
Figure 1. Geographical locations of 3 populations of *Vriesea cacuminis* at Minas Gerais State: **1** - Population 1 (Peão; range = 200 m); **2** to **5** - Population 2 (Cruzeiro: range = 3 km); **6** - Population 3 (Serra Negra: range = 150 m). The average distance between population 1 and population 2 is nearly 3 km. The average distance between 5 sampling points of the population 2 is 600 m and the distance between populations 1, 2 (Ibitipoca State Park), and 3 (Serra Negra) is approximately 30 km.

DNA extraction and PCR amplification

DNA extraction was performed according to Ferreira and Grattapaglia (1998), with

few modifications. Each DNA sample was quantified using a Nanodrop ND-100 spectrophotometer (Nanotrop, USA) and stored at -20°C. Initially, 30 anchored primers developed at University of British Columbia were tested. From those, 16 primers that showed good patterns of amplification were selected (Table 1).

Table 1. Primer sequence, number of loci detected/primer, and percentage of polymorphic bands (PPB) by inter-simple sequence repeat markers for *Vriesea cacuminis*.

Primer	Sequence (5'-3')	No. of loci detected	PPB
UBC-808	AGAGAGAGAGAGAGAGC	3 (2)	66.6
UBC-815	CTCTCTCTCTCTCTCTG	7 (7)	100.0
UBC-818	CACACACACACACACAG	8 (8)	100.0
UBC-820	GTGTGTGTGTGTGTGTC	2 (2)	100.0
UBC-823	TCTCTCTCTCTCTCTCC	5 (4)	80.0
UBC-824	TCTCTCTCTCTCTCTCG	4 (4)	100.0
UBC-827	ACACACACACACACACG	2 (2)	100.0
UBC-835	AGAGAGAGAGAGAGAGYC	5 (4)	80.0
UBC-841	GAGAGAGAGAGAGAGAYC	7 (6)	85.7
UBC-842	GAGAGAGAGAGAGAGAYG	6 (5)	83.3
UBC-844	CTCTCTCTCTCTCTCTRC	7 (6)	85.7
UBC-845	CTCTCTCTCTCTCTCTRG	6 (6)	66.6
UBC-848	CACACACACACACACARG	4 (3)	75.0
UBC-850	GTGTGTGTGTGTGTGTYC	8 (7)	87.5
UBC-851	GTGTGTGTGTGTGTGTYG	5 (4)	80.0
UBC-857	ACACACACACACACACYG	7 (7)	100.0

Numbers in parentheses refer to the number of polymorphic bands/primer.

ISSR markers were obtained according to Meloni et al. (2006), with minor modifications. The reaction was carried out in a volume of 25 μL containing 0.5 μM primer, 0.15 mM dNTPs, 1.0 U Taq DNA polymerase (Promega), 10 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 50 mM KCl, and 30 ng/μL template DNA. DNA amplification was performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Perkin Elmer) with the following conditions: 4 min at 94°C followed by 45 cycles of 1 min at 94°C, 45 s annealing at 50°C and a 2-min extension at 72°C, and finally an extension cycle of 7 min at 72°C. DNA fragments from PCR amplification were loaded on a 1.5% agarose gel and submitted to electrophoresis using 1X TBE buffer (100 V for 4 h), stained with 3 μg/mL ethidium bromide for 30 min and photodocumented with the EagleEye (Stratagene) system. The molecular weight of the fragments was estimated using a molecular marker ladder of 100 bp (Amresco).

Data analysis

Only consistent bands were used. ISSR bands were considered as present (1) or absent (0), and a binary qualitative matrix was made and analyzed using the GENES software (Cruz, 1998). These data were used to make a dissimilarity matrix based on the complement of the Jaccard index (*I*), determined as 1-S. The Jaccard index was calculated by the expression: $Sij =$ a / (a + b + c), with *a* being the number of bands shared by the sample pair (*i* and *j*), and *b* and *c* the number of bands present in samples *i* and *j*, respectively (Sneath and Sokal, 1973). A dendrogram was also constructed by an unweighted paired group method of cluster analysis using arithmetic averages (UPGMA). Bootstrapping based on the fingerprinting data was carried out using 1000 replicates. Analysis of molecular variance (AMOVA) was performed as described

by Excoffier (1992). Additionally, an estimate of the hierarchical variability was obtained by the Shannon index (Bussell, 1999) using the expression: H = -Σ Pi log2 Pi, where *Pi* is the frequency of a given ISSR band. The index was calculated for each locus (H_O). The average of all markers within a population was called H'pop and the average of H'pop within species was called Hpop. The average of all markers within species, without considering populations, was designated Hsp. The within-population component was calculated as (Hsp - Hpop) / Hsp, and the between-population component as Hpop/Hsp. The POPGENE software (Yeh et al., 1997) was used to estimate the Shannon index and calculate the percentage of polymorphic bands (PPB). PPB was used as descriptive information and was obtained dividing the number of polymorphic bands at the population and species levels by the total number of scored bands. A Bayesian analysis was performed using the STRUCTURE 2.3.3 software (Hubisz et al., 2009) to infer the number of genetic clusters (K). Ten independent runs were performed with a 100,000 burn-in period length and 500,000 Markov chain Monte Carlo replicates after burn-in, testing from 1 to 8 genetic clusters (K = 1-8). Parameter sets assumed the admixture model with alleles correlated between populations. We determined the average of each K likelihood value, 'log of probability' [LnP(D)] through all runs to infer the number of genetic clusters (populations) as suggested by Pritchard et al. (2000). The statistic Δ*K* was estimated according to Evanno et al. (2005).

RESULTS

The 16 selected ISSR primers yielded 86 steady markers in 70 individuals. The molecular weight ranged from 200 to 1100 bp. Table 1 summarizes the number of fragments and the PPB observed for each primer. The average number of bands per primer was 5.3 and the PPB ranged from 66 to 100%. Several markers were population-specific. Of 86 markers, 10 were observed only in 1 population while the others were observed in at least 2 populations (data not shown).

Table 2 summarizes the estimated diversity for each population and also for the species (without population structure). Population 2 (Cruzeiro) showed the highest level of variability. The PPB was 76.5%, the Shannon index was H = 0.31, and the mean of dissimilarity (D) among individuals was 0.3. Population 3 (Serra Negra) showed less diversity (PPB = 45.88%, H = 0.22, D = 0.21). Diversity was always lower at the population level than at the species level. The PPB for the species was 87.06% and the Shannon index was 0.33.

Table 2. Number of individuals (N) and genetic variation of *Vriesea cacuminis* populations.

V. cacuminis population	N	PPB	H	D
Population 1 (Peão)	20	68.24	0.29	0.26
Population 2 (Cruzeiro)	29	76.50	0.31	0.30
Population 3 (Serra Negra)	21	45.88	0.22	0.21
Species	70	87.06	0.33	0.29

PPB = percentage of polymorphic bands; H = Shannon index; D = dissimilarity average calculated using an average of the Jaccard index (1-S).

Clones were identified only in 2 individuals of Population 1 (Peão). The Jaccard similarity index was the same between 2 pairs of individuals collected side by side in the field. Therefore, it was possible to identify 68 different genotypes among 70 individuals investigated.

The partition of the variability - AMOVA - revealed significant differentiation (P < 0.001) between populations (about 16% from the total diversity). The remaining 84% was distributed between individuals within populations (Table 3). Similarly, by the Shannon diversity index, the estimated diversity within populations (Hpop/Hsp) was 81% and between populations [(Hsp - Hpop) / Hsp] was 19%.

Table 3. Analysis of molecular variance of *Vriesea cacuminis* populations.

Source of variance	d.f.	SSD	% of total variance	P value	Φ-statistics
Among populations	2	78.05	15.96	<0.001	$\Phi_{ST} = 0.1596$
Within populations	67	487.29	84.04	<0.001	$1-\Phi_{ST} = 0.8404$
Total	69	565.34	100.00		

d.f. = degrees of freedom; SSD = sum of squared deviations.

Even if the dendrogram constructed using the UPGMA algorithm with the similarity matrix based on the Jaccard coefficient (Sneath and Sokal, 1973) did not group the populations with high bootstrap support (data not shown), the cluster using Bayesian analysis (Evanno et al., 2005) indicated that K = 2 was the most likely structure of the populations. There was one major genetic cluster containing the populations from Ibitipoca Park (Peão and Cruzeiro) and another with populations from Serra Negra (Figure 2).
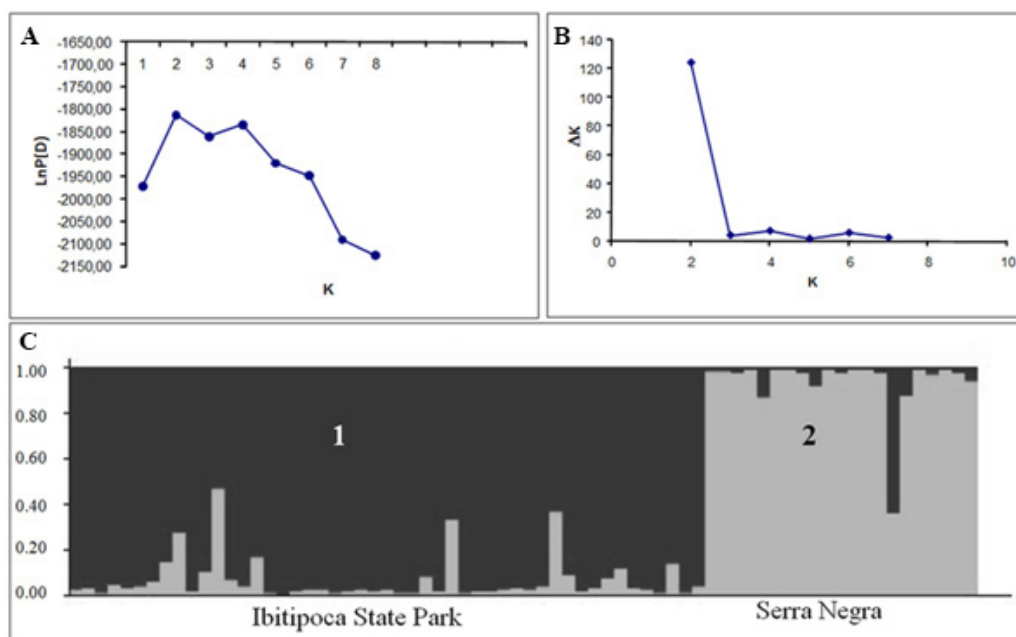
Figure 2. Bayesian analysis of 70 individuals from 3 populations of *Vriesea cacuminis*. **A.** Average log probability of data LnP(D) for each K of 10 independent runs (likely number of populations = 2). **B.** Average ΔK for each K of 10 independent runs (likely number of populations = 2). **C.** Graphic representation of the different genetic pools for K = 2. The first genetic group (1) is depicted in black (most of individuals from Ibitipoca range) and the second group (2) is depicted in gray (most of individuals from Serra Negra range).

DISCUSSION

Ibitipoca State Park is a protected area with 1488 ha that hosts a great number of endemic species, including some that belong to the family Bromeliaceae (Monteiro and Forzza, 2008). Despite the large diversity, few studies about the genetic diversity and population structure of these species have been carried out.

Considered as a rare, restricted and endemic species, *V. cacuminis* has a relatively high genetic diversity assessed by ISSR markers (PPB = 87% and I = 0.33%). Although higher genetic diversity has been mainly related to wider distributed species (Souza and Lovato, 2010), geographically restricted species have also shown relatively high diversity index (Cavallari et al., 2006; Huang et al., 2008). Consequently, these data provide evidence that other factors, in addition to geographical distribution, could contribute to maintain the variability in rare and restricted species.

As was already observed for other species, including the Bromeliaceae, the level of diversity varies according to population size (Hmeljevski et al., 2011). For *V. cacuminis*, Population 2 (Cruzeiro), which was the largest population, showed the highest genetic diversity (PPB = 76.5%, I = 0.31, D = 0.3). On the other hand, the lowest values of genetic diversity (PPB = 45.88%, I = 0.22, D = 0.21) were observed for Population 3 (Serra Negra), which had the smallest number of individuals concentrated on a rock wall 150 m long. Population 1 (Peão) showed intermediate values of diversity, as compared to the others, and it was formed by individuals scattered on a steep rocky outcrop.

The capacity of dispersion and the reproduction system are also important factors in determining the structure and the diversity of plant populations. Previous studies have suggested that a significant difference between 2 populations occurs when the rate of genetic differentiation (e.g., Gst or Φst) is higher than 0.25 (Slatkin, 1987; Han et al., 2007). Our data for *V. cacuminis* do not suggest considerable distinction between the 3 populations, since genetic differentiation between populations, indicated by Φst, was only 0.16. Similarly, using the UPGMA algorithm, it was not possible to observe clusters among populations with high bootstrap support, which suggests the absence of a clear differentiation between the 2 populations of the Ibitipoca State Park (Peão and Cruzeiro) and also between these populations and the Serra Negra population. On the other hand, Bayesian clustering analysis showed 2 distinct clusters among Ibitipoca Park and Serra Negra populations. AMOVA also indicated that part of the diversity is distributed among populations, supporting the split of the individuals into 2 major groups (Ibitipoca and Serra Negra).

Regarding plant reproductive strategies, a mixed system of vegetative reproduction and outcrossing, commonly observed in species of Bromeliaceae (Benzing, 2000), also seems to be responsible for maintaining the population structure of *V. cacuminis*. Field observations revealed evidence of vegetative propagation and ramets, which are typical of clonal reproduction (Callaghan et al., 1990; Versieux, 2011). Individuals with identical ISSR markers (probably clones) in Population 1 (Peão) suggest that individuals isolated by short distances (<1 m) could be previously linked. This fact has also been reported for another species of the Bromeliaceae, *Encholirium biflorum* (Mez) Forzza, which possesses a clonal habit and occurs isolated or in small clumps spread out on sandy-rocky soil (Cavallari et al., 2006). However, these observations for *E. biflorum* and *V. cacuminis* may not be very common. The variability among populations of *V. cacuminis* ranged from 16 to 19%, also suggesting the occurrence of

outcrossing. Unfortunately, there are no data about the reproductive system of *V. cacuminis*. Autocompatibility is predominant within the genus (e.g., Siqueira-Filho, 2006; Matallana et al., 2010), although autoincompatibility has also been reported (Matallana et al., 2010). Regardless of the existence of an autoincompatibility mechanism in *V. cacuminis*, the flowers are adapted to pollination by hummingbirds (tubular flowers, with conspicuous color, no odor and large nectar production) and pollen flow could occur even if selfing is detected (Linhart et al., 1987; Parra et al., 1993). Wind dispersal, which occurs in *V. cacuminis*, in addition to cross-breeding, can help to decrease the genetic differentiation between the patches along the range as was observed along the Cruzeiro range. The high levels of diversity estimated within each population could be explained by clone longevity, as described for other clonal species (Esselman et al., 1999; Cavallari et al., 2006).

It is possible that Populations 1 (Peão) and 3 (Serra Negra) had been founded by a small number of seeds from Population 2 (Cruzeiro). The higher similarity estimated among individuals of Serra Negra, compared to that found among individuals from the other populations, suggests a lower number of events of seed dispersal to Serra Negra. Possibly, the gene flow between the populations of the 2 ranges can be maintained by hummingbirds or bats, which are the most common pollinators of the Bromeliaceae (Benzing, 2000). This dispersal of pollen could also prevent the differentiation of *V. cacuminis* populations along those 2 hills. Nevertheless, we cannot rule out the possibility that these 2 areas had been recently colonized, and therefore the elapsed time has not yet permitted significant differentiation between those populations and Population 2 (Cruzeiro). Another hypothesis could be that those populations (from Ibitipoca and Serra Negra) were connected in the past forming one major continuous population. In this case, the time of isolation might not have been enough to allow high levels of genetic differentiation.

Considering that the amount of genetic diversity has a straightforward relationship with the evolutionary potential of one species, understanding the population structure is essential to take effective measures of variability management and conservation. Our results showed that it was possible to detect higher diversity indices of *V. cacuminis* populations at Ibitipoca State Park (protected area) than Serra Negra (unprotected area). The genetic similarity of all the populations investigated revealed that these populations are probably not isolated. Regarding the populations from Ibitipoca State Park, the larger number of genotypes within populations, and also the higher genetic variability in Population 2 (Cruzeiro) indicate this area as an important source of genetic variability. Moreover, in addition to the higher variability, the large number of different individual genetic profiles also indicates that a great number of individuals should be collected to get a representative germplasm collection.

Although the present results help to explain how *V. cacuminis* populations keep their variability, it is important to check how far other processes such as self-fertilization and vegetative reproduction have affected the population structure.

In conclusion, despite restricted distribution, *V. cacumins* exhibited relatively high diversity within populations. The populations are located up to 30 km between each other and do not seem to be structured, perhaps as a consequence of sexual and asexual reproduction occurring simultaneously. Cross-breeding probably maintains the gene flow between the 3 existing populations and therefore the low level of differentiation between them. In each population, the longevity of clones probably contributes to maintaining different genotypes and increasing diversity within groups. The widest variability was detected within Ibitipoca State Park, whereas Serra Negra exhibited an outlying population, with less genetic variability.

ACKNOWLEDGMENTS

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