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Cross-amplification and characterization of microsatellite markers in *Alcantarea patriae* Versieux & Wand.

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ABSTRACT. The *Alcantarea patriae* is a Bromeliaceae endemic to the inselbergs of the Atlantic Forest. This taxon, described in the year of 2007 by Versieux & Wanderley, presents restricted and fragmented distribution outside conservation units. Studies to evaluate the genetic structure of its populations can contribute to the conservation and management strategies for the species. In this study, 31 microsatellite markers, descriptive to six different Bromeliaceae species, were evaluated by cross-amplification tests in 20 individuals of *A. patriae*.

The individuals were collected in the district of Vila Cruzeiro, in the municipality of Jerônimo Monteiro. Twelve markers were polymorphic and 10 monomorphic, with an amplification success rate of 71%. The displayed polymorphism information content was considered high, indicating that the selected markers are informative. The values found for the fixation index were positive and indicated the occurrence of inbreeding. The mean number of alleles was 4.66 (3-6), the mean expected and observed heterozygosities were 0.6605 and 0.4618, respectively. The detection of polymorphic markers was important for future studies of diversity and genetic structuring of natural populations and for germplasm bank creation aiming to contribute to *in situ* and *ex situ* conservations of *A. patriae*.

Key words: Transferability; SSR; Atlantic Forest; Bromeliaceae

INTRODUCTION

The genus *Alcantarea* belongs to the family Bromeliaceae and subfamily Tillandsioideae and includes about 26 species occurring in the Atlantic Forest (Versieux et al., 2010). In 2007, Versieux and Wanderley described a new species of bromeliad denominated *Alcantarea patriae* Versieux & Wand. This species naturally occurs in small fragmented populations occurring exclusively in some inselbergs in the Southern State of Espírito Santo (Versieux and Wanderley, 2007). These sites are located on private properties outside conservation units and are subject to several types of impacts, such as fire degradation, invasion of exotic species and extractive collection (Manhães et al., 2016).

A. patriae has some ecological and economic characteristics attractive to landscaping, such as rusticity, beauty, the practicality of cultivation, and durability of its inflorescence and its flowers that present green/yellow coloring (Versieux, 2009). Ornamental interest encourages the illegal extraction of this species in natural populations (Versieux and Wanderley, 2007), which combined with other factors such as limited geographic distribution, reduced population size, and environmental degradation may promote genetic loss.

One way to obtain data on genetic diversity is through the use of microsatellite or SSR markers (Litt and Luty, 1989). These markers are distributed throughout the genome of eukaryotes, are multi-allelic, with high variability and codominant pattern (Müller et al., 2010).

Despite the great usefulness and informativeness in genetic studies, obtaining SSR markers is a process that demands time and high financial investments. In this way, the development of species-specific primers becomes a limitation to the use of microsatellites for genetic analysis in several species (Barbará et al., 2007). An alternative way to use this technique is the transferability of the markers, which makes it possible to carry out population studies in species for which SSR markers have not yet been described (Zhang and Hewitt, 2003; Barbará et al., 2007).

Thus, the present study aimed to evaluate and characterize the transferability of microsatellite markers, originally developed for *Alcantarea imperialis* Carrière Harm., *Guzmania monostachya* Rusby ex Mez., *Pitcairnia geyskesii* LB Smith, *P. albiflos* L'Her, *Tillandsia fasciculata* Swartz, and *Vriesea gigantea* Gaud., in DNA samples from *Alcantarea patriae* Versieux & Wand.

MATERIAL AND METHODS

Leaf samples were collected from 20 adult *A. patriae* individuals from the population located in the district of Vila Cruzeiro (20°47'46"S, 41°21'55"W) in the municipality of Jerônimo Monteiro. Total DNA was extracted by the cetyltrimethylammonium bromide method (Doyle and Doyle, 1990) at the Laboratory of Biochemistry and Molecular Biology of Universidade Federal do Espírito Santo. After extraction, the quantification was performed, and the purity of the DNA obtained was checked through the use of NanoDrop®.

In all, 31 microsatellite markers described by different authors for different species of Bromeliaceae were evaluated in transferability tests (Table 1).

The e6 and e6b *loci* were amplified in reactions containing 10 mM Tris/KCl, pH 8.5, 1.5 mM $MgCl_2$, 1 U Taq DNA polymerase, 0.1 mM dNTP, 30 ng DNA, and 3 µM of each primer forward (F) and reverse (R). The PCR program consisted of a denaturation step of 94°C for 3 min, 44 cycles of 94°C for 20 s, 40 s at the annealing temperature (Table 2) and 72°C for 20 s. The extension phase occurred for 5 min at 72°C. The best results for Pit6 and PaZ01 *loci* were obtained by amplification in reactions containing 10 mM Tris/KCl buffer, pH 8.5, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1 U Taq DNA polymerase, 0.2 µM of each primer (F and R), and 30 ng DNA. The PCR conditions differed from the conditions proposed and consisted of 94°C for 5 min, 30 cycles at 94°C for 1 min, annealing temperature (Table 2) for 1 min and 72°C for 1 min, and a final extension phase of 72°C for 7 min.

Table 1. Microsatellite markers used in transferability assessment.

Species	Author	SSR markers
Alcantarea imperialis	Palma-Silva et al., 2007	Ai4.03, Ai4.11, Ai4.10, Ai5.18
Guzmania monostachya	Boneh et al., 2003	CT5
Pitcairnia albiflos	Paggi et al., 2008	PaA05, PaA9, PaA10, PaB11, PaB12, PaC05, PaD07, PaZ01
Pitcairnia geyskesii	Sarthou et al., 2003	Pit2, Pit4, Pit5, Pit6, Pit8, Pit9, Pit 12
Tillandsia faciculata	Boneh et al., 2003	e6, p2p19, e19, e6b
Vriesea gigantea	Palma-Silva et al., 2007	VgA04, VgB06, VgB10, VgC01, VgF02, VgG03, VgG05

Table 2. Polymorphic microsatellite markers selected in *Alcantarea patriae*.

Loci	Fragments size	Ta (°C)	N_A	Brookfield 1**	PIC	H_E	H_O	F_{IS}	H’	R_A
Pit6	168-178	55	5	Ns	0.721	0.760	0.666	0.214*	0.778	4.928
PaA10	151-178	58-48	6	0.3629	0.719	0.754	0.117	0.853*	0.798	5.891
PaD07	228-234	58-48	3	0.2705	0.393	0.476	0.076	0.850*	0.513	3.000
PaZ01	210-226	57	6	Ns	0.702	0.745	0.736	0.040*	0.768	5.564
e6	140-170	51	5	Ns	0.739	0.77	0.70	0.122*	0.797	4.996
e6b	142-157	51	3	Ns	0.539	0.608	0.60	0.040*	0.625	3.000
VgB10	138-164	58-48	6	0.1278	0.783	0.810	0.578	0.310*	0.839	5.985
VgC01	150-164	58-48	4	Ns	0.589	0.646	0.631	0.051*	0.665	3.906
VgG03	223-255	58-48	5	Ns	0.694	0.731	0.55	0.272*	0.755	4.985
VgG05	202-222	58-48	4	Ns	0.568	0.613	0.533	0.164*	0.638	3.999
Ai4.10	192-218	58-48	4	0.095	0.359	0.381	0.25	0.367*	0.395	3.836
Ai5.18	200-250	58-48	5	0.322	0.589	0.622	0.10	0.847*	0.653	4.946
Average	-	-	4.66	-	0.616	0.6605	0.4618	0.344	0.685	4.336

Ta = annealing temperature; N_A = number of alleles per *locus*; PIC = polymorphism information content; H_E = expected heterozygosity; H_O = observed heterozygosity; F_{IS} = inbreeding coefficient; H’ = gene diversity; R_A = allele richness; Ns = not significant. *All indices of fixation were positive and significant (probability of 0.00417%). **Brookfield 1, parameter used to measure the presence of null alleles.

For the *loci* PaA10 and PaD07, reactions were performed containing 20 ng DNA in a buffer solution with 10 mM Tris/KCl, pH 8.5, and 2 mM $MgCl_2$, 0.1 mM dNTP, 1 U Taq DNA polymerase, and 0.2 µM of each primer (F and R). PCRs performed with the VgC01, VgG03, and Ai4.10 markers contained 10 mM Tris/KCl, pH 8.5, 2.0 mM $MgCl_2$, 0.1 mM dNTP, 1 U Taq DNA polymerase, 30 ng DNA, and 0.4 µM of each primer (F and R). For VgG05 and Ai4.03 *loci*, the same PCR concentrations as those mentioned above were used, except for the concentration of $MgCl_2$ (1.5 mM). The best results for the VgB10 *locus* were obtained with Master Mix Promega. The GoTaq® Colorless Master Mix pre-mix used was composed of 1X Colorless GoTaq® buffer, pH 8.5, 0.2 mM of each dNTP, 1.5 mM $MgCl_{2,}$ 1 U Taq DNA polymerase, 0.4 mM of each primer (F and R), and 30 ng DNA. For all these markers the PCRs were performed in a touchdown program proposed by Palma-Silva et al. (2007), consisting of 95°C for 3 min, 10 cycles at 94°C for 30 s, 58°C falling to 48°C, 1°C per cycle for 30 s, 72°C for 30 s, followed by 30 cycles of 94°C for 30 s, 72°C for 30 s, and a final extension phase of 72°C for 10 min.

In all cases, the reactions had a final volume of 15 µL, and the PCRs were performed on the Applied Biosystems Veriti® 96-Well Thermal Cycler. The amplified fragments were separated by electrophoresis on 10% polyacrylamide gel at 110 V for approximately 3.5 h and stained with 0.02 µL/mL ethidium bromide solution. The images were analyzed for the number and size of amplified fragments for the detection of polymorphisms.

Initially, the data were evaluated for the detection of genotyping and null allele errors through the Brookfield 1 estimator using Micro-Checker v. 2.2.3 (Van Oosterhout et al., 2004). For the population analyses, the allele number (N_A) and allele richness (R_A) were estimated through the FSTAT v. 2.9.3.2 (Goudet, 2001). The expected heterozygosity (H_E), observed heterozygosity (H_O), polymorphism information content (PIC), and Hardy-Weinberg equilibrium (HWE) deviations were obtained with the Genes program (Cruz, 2013). The genetic diversity (H') and fixation index (F_{IS}) used the FSTAT v. 2.9.3.2 program (Goudet, 2001).

RESULTS AND DISCUSSION

The transferability of 31 microsatellite markers was evaluated, and after optimization of PCRs 12 loci generated polymorphic amplification products (Table 2), and 10 behaved as monomorphic (Pit4, Pit8, p2p19, e19, CT5, VgA04, VgB06, VgF02, Ai4.03, and Ai4.11).

Overall success in transferability was approximately 71% (22 of the 31 *loci* evaluated), with 54.5% of the markers transferred (12 of 22 loci) being polymorphic. Barbará et al. (2007) showed that the best results of polymorphic microsatellite markers in plants occur among the genera of the same subfamily, with a success rate of 40% in monocotyledons, but in Bromeliaceae, heterologous amplification is possible even among subfamilies. In the present study, the rate of polymorphic markers transferred within the same subfamily (Tillandsioideae) was 50% and among subfamilies (Pitcairnioideae to Tillandsioideae) was 26.6%, that is, four of the 15 tested. The success rate in cross-amplification in *A. patriae* was in agreement with the values obtained by other studies developed with Bromeliaceae species (Palma-Silva et al., 2007; Paggi et al., 2008; Miranda et al., 2012; Lavor et al., 2013).

The polymorphic *loci* had a mean N_A of 4.66 (3-6), H_E of 0.6605, and H_O of 0.4618. Four of the *loci* studied (PaZ01, e6, e6b, and VgC01) were in agreement with the HWE and in five *loci* the presence of null alleles was detected (Table 2). This fact could be related to the reduction of polymorphisms and the HWE deviations observed for these markers (Martins

et al., 2008). However, this information does not preclude the use of these *loci* for genetic studies.

The PIC presented was considered high, indicating that the selected markers were informative and indicated for later genetic studies. The values found for the F_{IS} were positive and ranged from 0.04 to 0.853 with a mean of 0.344. The mean H' was 0.685, and the R_A was 4.336 (Table 2).

We concluded that the markers obtained by the transferability were highly informative and presented a high transfer success rate. Thus, they are suitable for future genetic analyses, such as population studies that can evaluate genetic variability in natural populations. These studies may be important indicators for the establishment of conservation and management strategies, as well as contributing to the creation of germplasm bank, contributing to *ex situ* and *in situ* conservations of *A. patriae*.

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