Development, characterization, and transferability of SSR markers for *Vriesea carinata* (Bromeliaceae) based on RNA sequencing

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PREMISE OF THE STUDY: Expressed sequence tag–simple sequence repeat (EST-SSR) markers were isolated for *Vriesea carinata*, an endemic bromeliad from the Brazilian Atlantic Forest. These SSR loci may be used to investigate the genetic diversity and population structure of this species and related bromeliads.

METHODS AND RESULTS: Based on the transcriptome data of *V. carinata*, 30 primer pairs were designed and selected for initial validation. Of these primer pairs, 16 generated suitable SSR loci in 69 individuals. The number of alleles per locus ranged from one to 13; the levels of observed and expected heterozygosity per locus ranged from 0.000 to 1.000 and from 0.000 to 0.935, respectively. All loci produced heterologous amplification. Transferability of the loci was tested in 15 species belonging to three Bromeliaceae subfamilies.

CONCLUSIONS: The developed EST-SSR markers revealed polymorphism in the four studied populations and could be useful to investigate the genetic diversity of *V. carinata* and related species. The markers may also be suitable for novel gene annotation and discovery.

KEY WORDS: Aechmea; Alcantarea; *Bromelia*; Bromeliaceae; Dyckia; microsatellite markers; Neoregelia; RNA-Seq; *Vriesea carinata*.

Bromeliaceae is a Neotropical angiosperm family comprising approximately 58 genera and 3352 species distributed in eight subfamilies (Luther, 2012). *Vriesea* Lindl. is part of the monophyletic Tillandsioideae subfamily and is the third largest genus endemic to Brazil, with the Atlantic Forest being its primary center of diversity (Barfuss et al., 2016). *Vriesea carinata* Wawra is an epiphyte or terrestrial species that exists in mesopholic environments and well-preserved habitats with high humidity distributed along the Atlantic Forest. This species is pollinated by hummingbirds and often used as an ornamental plant, which causes them to be illegally extracted. The increased extractivism of this species, linked to the fragmentation of the Atlantic Forest, has caused concern with respect to the conservation of *V. carinata*, as well as many other bromeliad species (Zanella et al., 2012).

To date, slightly more than a dozen studies have characterized simple sequence repeat (SSR) loci in Bromeliaceae. Of these studies, only three have investigated the subfamily Tillandsioideae (Boneh et al., 2003; Palma-Silva et al., 2007; Neri et al., 2015), and no loci have been tested for cross-amplification in *V. carinata*. Considering the high rate of transferability of SSR loci within the same subfamily in Bromeliaceae (Zanella et al., 2012; Goetze et al., 2013; Neri et al., 2015), the development of SSRs in Tillandsioideae may provide markers that can be used in a range of species and that will be valuable tools in the study of species delineation, phylogeography, variation in mating systems, and the detection of hybridization and introgression (Zanella et al., 2012). In this report, we describe the development of 16 expressed sequence tag (EST)–SSR markers for *V. carinata*.
| Locus  | Primer sequences (5'–3')               | Repeat motif                  | Allele size range (bp) | Putative function [Organism]                                      | E-value | GenBank accession no. |
|--------|----------------------------------------|--------------------------------|------------------------|-------------------------------------------------------------------|---------|----------------------|
| Vcar_10 | F: AACTTGCGCTATGCTCAAAGGAAATGGG        | (TTG)_3(N(TTT))_3(N(TAT))_2   | 243–261                | Endoribonuclease Dicer homolog 1 isoform X1 [Ananas comosus]       | 0.0     | MF563945             |
|        | R: ACTCTCGGCTGTTCTCTCCTCCTCCTC         |                               |                        |                                                                    |         |                      |
| Vcar_31 | F: CCGTAGGCGAGGACAGATAAGAGGG           | (AG)_15                       | 206–236                | E3 SUMO-protein ligase SIZ1 [Ananas comosus]                       | 0.0     | MF563947             |
|        | R: GTGGGAGGAGGAGAGAGAGAGAGAGAGAGAGAAG  |                               |                        |                                                                    |         |                      |
| Vcar_36 | F: CCGAGGCTCTCTCCCTTTCC                | (CGC)_9                       | 262–265                | Flowering time control protein FPA [Ananas comosus]                | 0.0     | MH319796             |
|        | R: TCTATCCGACTCCTTTGTCTGTGTCGTCTGT     |                               |                        |                                                                    |         |                      |
| Vcar_72 | F: TCCTGGCTTCTCCCGGCAAGA               | (CGC)_3(N(GTCC)T)_5(N(TCC))_6 | 206–237                | Histone acetyltransferase GCN5 [Ananas comosus]                   | 0.0     | MH363948             |
|        | R: GGAACCCACCTGTTGGTCAG TCCT            |                               |                        |                                                                    |         |                      |
| Vcar_91 | F: TTAGGCTGTTGGGGGTCTC                 | (AG)_3                        | 186–213                | Adagio-like protein 3 [Ananas comosus]                            | 0.0     | MF563949             |
|        | R: AGATGGCTGAGGAAGTGCAGA                |                               |                        |                                                                    |         |                      |
| Vcar_93 | F: TTGCACAAAGACGTACCAAA                | (AG)_3                        | 229–259                | Coronatine-insensitive protein homolog 1b-like [Ananas comosus]    | 0.0     | MF563950             |
|        | R: TGCTGGGAAGAAGGTACACTA                |                               |                        |                                                                    |         |                      |
| Vcar_95 | F: CATTGGTGTGTGGGTTTC                   | (TGCCAC)_6                    | 200–224                | Calmodulin-like [Gossypium raimondii]                              | 1e-97   | MF563951             |
|        | R: GAGATGGCTGAGGAAGAAGATG               |                               |                        |                                                                    |         |                      |
| Vcar_115 | F: GCCCATATCACAAGCATACA               | (GA)_2(N(AG))_2              | 200–222                | Leucine-rich repeat receptor-like serine/threonine/tyrosine-protein kinase [Ananas comosus] | 6e-171 | MF563952             |
|        | R: GAGGCTATTTTATGTCTCTGTC               |                               |                        |                                                                    |         |                      |
| Vcar_139 | F: CCCCGAATTTGCTGAAAC                | (AGG)_3(N(TGG))_5(N(AGA))_9   | 246–264                | Homogentisate phytyltransferase 2, chloroplastic isoform X1 [Ananas comosus] | 0.0     | MF563953             |
|        | R: GGATGATGAGATTTGCGGGTTTG              |                               |                        |                                                                    |         |                      |
| Vcar_143 | F: CCCCCTCAGGTCAATTTTAT                | (CTC)_5(N(TAGGGT))_2         | 240–264                | Probable serine/threonine-protein kinase WNK4 [Elaeis guineensis]  | 4e-86   | MF563954             |
|        | R: AGGAGATGGCCATGCAAACC                 |                               |                        |                                                                    |         |                      |
| Vcar_153 | F: CTTCCAGGAGGCAGAGCAAA               | (GCTGAA)_3                   | 222–234                | Copper transport protein ATX1 [Phalaenopsis equestris]             | 7e-18   | MF563955             |
|        | R: AGTACAAATGCTGACGCTGTCGGGGGG          |                               |                        |                                                                    |         |                      |
| Vcar_258 | F: TTCTCGAGGACCTGCGATCC                | (CCT)_7                      | 228–237                | Monothiol glutaredoxin-S14, chloroplastic [Ananas comosus]         | 4e-96   | MF563956             |
|        | R: GAGTCATGGCGGAGGAAGAATG              |                               |                        |                                                                    |         |                      |
| Vcar_2801 | F: CACAAGAGCAGCATACAAACCC              | (CTC)_6                      | 220–248                | Basic blue protein-like isoform X1 [Ananas comosus]               | 4e-78   | MF563957             |
|        | R: ACTGCAATCCGCGGATGAAAG               |                               |                        |                                                                    |         |                      |
| Vcar_293 | F: TCTGGGACTCTAGGGTTTGT                | (CCT)_6                      | 244–260                | DNA mismatch repair protein PM51 isoform X3 [Ananas comosus]       | 2e-142  | MH319797             |
|        | R: CACAAGACTCTTCAGACGAG                 |                               |                        |                                                                    |         |                      |
| Vcar_395 | F: CTTGATATGTCCTGGTCGCTTC              | (TTG)_3                      | 224                    | Transcription factor HYS-like [Ananas comosus]                     | 2e-91   | MH319798             |
|        | R: CGATGATGTCGCTGCCGCCGCGAGCTGGTGA     |                               |                        |                                                                    |         |                      |
| Vcar_501 | F: GATTTGATGCGCGGAGGATGTAGA            | (AGAA)_3                     | 130                    | MADS-box protein JOINTLESS-like [Ananas comosus]                  | 3e-30   | MH319799             |
|        | R: ACGATGATGACCCGATCCAGTGG             |                               |                        |                                                                    |         |                      |

*All loci except Vcar_31 and Vcar_36 were amplified using a touchdown program with a temperature range between 58°C and 48°C (Palma-Silva et al., 2007).*
using next-generation transcriptome sequencing. Additionally, we tested the transferability of these markers to other bromeliad species.

**METHODS AND RESULTS**

Total RNA was isolated from *V. carinata* leaves as described by Guzman et al. (2013). RNA quality was then assessed by 1% agarose gel electrophoresis and quantified by the Thermo Scientific NanoDrop Lite Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). Subsequently, an RNA-Seq library was sequenced using the Illumina HiSeq 2000 platform (Illumina, San Diego, California, USA) by Fasteris (Geneva, Switzerland) and assembled de novo in transcripts using the CLC Genome Workbench version 4.0.2 (QIAGEN, Hilden, Germany) as reported by Guzman et al. (2013). All sequence information was deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA; PRJNA167588). From this library, a preliminary analysis of the presence of microsatellites and the determination of the functions of each fragment was undertaken using the Blast2GO tool (Conesa et al., 2005), which generated a draft of potential sequences for the isolation of microsatellites. Preliminary sequences were analyzed, and the primers encompassing the microsatellite regions were designed using Primer3 software (Koressaar and Remm, 2007). Forward primers were synthesized with a 19-bp M13 tail (5′-CACGACGTGTAAACGAC-3′) at the 5′ end to enable labeling with a tagged fluorescent dye (FAM, NED, VIC, or PET) during amplification and multiplex genotyping procedures.

To test isolated SSR loci, fresh leaves were collected from 69 individuals from four natural populations of *V. carinata* (Appendix 1) and stored in silica gel. Total genomic DNA was extracted according to Doyle and Doyle (1990). The PCR reactions were performed in a final volume of 10 μL, containing 10 ng of genomic DNA, 1× enzyme buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 10 μM of each primer (forward and reverse), and 0.5 unit of *Taq* polymerase (GoTaq; Promega Corporation, Madison, Wisconsin, USA). The amplification protocol was performed according to Palma-Silva et al. (2007), with the exception of loci Vcar_31 and Vcar_36. The PCR reaction for these loci was performed with an initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s; and a final extension of 72°C for 10 min. PCR was repeated for loci with positive amplification by adding the universal M13 primer labeled with different fluorochromes (FAM, VIC, PET, or NED) according to Goetze et al. (2013). The amplification products were genotyped in an ABI 3500 DNA Genetic Analyzer (Applied Biosystems, Foster City, California, USA) and compared to GeneScan 500 LIZ Size Standard, with allele identification performed in GeneMarker software Demo version 1.97 (SoftGenetics, State College, Pennsylvania, USA). To describe the variation of the microsatellites, observed and expected heterozygosities were estimated for each locus and population using the software MSA (Dieringer and Schlötterer, 2003). FSTAT (Goudet, 1995) was used to estimate the number of alleles, and GENEPOP ON THE WEB (Raymond and Rousset, 1995) was used to test for Hardy–Weinberg equilibrium and linkage disequilibrium and to estimate the inbreeding coefficient ($F_{is}$).

| Loci | Morretes (n = 21) | Santa Virginia (n = 24) | Bertioga (n = 11) | Ubatuba (n = 13) |
|------|------------------|------------------------|------------------|------------------|
| Vcar_10 | 7 | 0.762 | 0.811 | 0.062 | 2 | 0.000 | 0.667 | 1.000 | 7 | 0.636 | 0.792 | 0.204 | 7 | 1.000 | 0.837 | −0.210 |
| Vcar_31 | 9 | 0.444 | 0.869 | 0.504 | 13 | 0.667 | 0.927 | 0.287 | 7 | 0.500 | 0.837 | 0.416 | 4 | 0.200 | 0.778 | 0.765 |
| Vcar_36 | 2 | 0.190 | 0.176 | 0.081 | 2 | 0.043 | 0.434 | 0.0 | NT | NT | NT | 4 | 0.000 | 0.000 | — | 1 | 0.000 | 0.000 | — |
| Vcar_72 | 4 | 0.263 | 0.633 | 0.591 | 4 | 0.125 | 0.642 | 0.816 | 3 | 0.300 | 0.652 | 0.554 | 4 | 0.363 | 0.636 | 0.441 |
| Vcar_91 | 5 | 0.176 | 0.451 | 0.616 | 2 | 0.062 | 0.626 | 0.0 | 1 | 0.000 | 0.000 | — | 3 | 0.600 | 0.611 | 0.018 |
| Vcar_93 | 12 | 0.809 | 0.883 | 0.085 | 12 | 0.545 | 0.935 | 0.429 | 11 | 0.909 | 0.999 | 0.000 | 11 | 0.833 | 0.913 | 0.091 |
| Vcar_95.1 | 7 | 0.667 | 0.756 | 0.121 | 3 | 1.000 | 0.733 | −0.500 | 5 | 0.454 | 0.679 | 0.342 | 5 | 0.727 | 0.749 | 0.030 |
| Vcar_115 | 7 | 0.550 | 0.608 | 0.097 | 5 | 0.353 | 0.684 | 0.492 | 3 | 0.364 | 0.554 | 0.355 | 8 | 0.444 | 0.745 | 0.418 |
| Vcar_139 | 5 | 0.375 | 0.542 | 0.165 | 1 | 0.000 | 0.000 | — | 4 | 0.000 | 0.800 | 1.000 | 5 | 0.500 | 0.768 | 0.359 |
| Vcar_143 | 6 | 0.428 | 0.449 | 0.048 | — | — | — | — | 3 | 0.400 | 0.542 | 0.273 | 6 | 0.833 | 0.717 | −0.170 |
| Vcar_153 | 5 | 0.333 | 0.561 | 0.412 | 3 | 0.350 | 0.509 | 0.318 | 4 | 0.454 | 0.762 | 0.415 | 2 | 0.308 | 0.443 | 0.314 |
| Vcar_258 | 3 | 0.571 | 0.648 | 0.122 | 2 | 0.350 | 0.667 | 1.000 | 3 | 0.428 | 0.659 | 0.368 | 3 | 0.500 | 0.750 | 0.368 |
| Vcar_280.1 | 9 | 0.067 | 0.811 | 0.920 | 3 | 0.000 | 0.800 | 1.000 | 4 | 0.000 | 0.747 | 1.000* | 9 | 0.500 | 0.908 | 0.467 |
| Vcar_293 | 3 | 0.450 | 0.619 | 0.278 | 5 | 0.500 | 0.833 | 0.423 | NT | NT | NT | NT | NT | NT | NT |
| Vcar_395 | 1 | 0.000 | 0.000 | — | 1 | 0.000 | 0.000 | — | NT | NT | NT | NT | NT | NT | NT |
| Vcar_501 | 1 | 0.000 | 0.000 | — | 1 | 0.000 | 0.000 | — | NT | NT | NT | NT | NT | NT | NT |
| Mean | 5.375 | 0.380 | 0.570 | 0.310 | 4.143 | 0.260 | 0.536 | 0.526 | 4.583 | 0.370 | 0.661 | 0.448 | 5.583 | 0.567 | 0.738 | 0.241 |

*Note:* — index could not be calculated; $A$ = number of alleles; $F_{is}$ = inbreeding coefficient; $H_e$ = expected heterozygosity; $H_o$ = observed heterozygosity; $N$ = number of plants sampled; NT = locus not tested for the population (due to problems with the DNA of some individuals, the last isolated loci could not be tested in these populations).

*Locality and voucher information are provided in Appendix 1.*

*Inbreeding coefficient ($F_{is}$) departed significantly from Hardy–Weinberg equilibrium ($P < 0.001$).*
disequilibrium could not be tested by Fisher’s test in Vcar_501 and Vcar_395 and in three pairs of loci with Vcar_139 (Vcar_31, Vcar_93, and Vcar_258). The 16 SSRs were tested in other bromeliads in order to verify their potential for heterologous amplification. Of the 16 characterized loci, seven amplified in more than 50% of the species (Table 3). The locus Vcar_36 amplified in all species followed by Vcar_501 (14), Vcar_153 (13), Vcar_258 (9), and Vcar_115/Vcar_143/Vcar_395, with each amplifying in eight species. All 16 loci amplified in V. reitzii Leme & A. F. Costa, and approximately 81% amplified in all five individuals tested (Table 4). These loci also amplified in some species belonging to subfamilies other than Tillandsioideae, which suggests their potential utility in genetic studies of populations involving other bromeliad subfamilies.

All markers were tested for their transferability in one individual of 15 species belonging to three Bromeliaceae subfamilies. The conditions of the PCR amplifications were the same as described above. The amplification products were run on 2.0% agarose gel electrophoresis, stained with GelRed (Biotium, Hayward, California, USA), and compared to 100-bp and 50-bp ladders (Ludwig Biotechnology Ltda., Alvorada, Rio Grande do Sul, Brazil). The loci were considered to have positive amplification when at least one band of the expected size was visualized. Additionally, cross-amplification was tested in five individuals of V. reitzii to assess potential individual variation. Voucher information concerning the species investigated is listed in Appendix 1.

CONCLUSIONS

The 16 SSR markers described in this study revealed polymorphism in the studied populations of V. carinata and can be useful for the study of genetic diversity and evolution in other related species. Additionally, the loci described in this study may be used in studies that promote conservation and management of V. carinata, which is increasingly threatened by extractivism and habitat destruction. Moreover, due to association with coding sequences, the EST-SSRs isolated in this study have the potential for direct gene tagging and can facilitate future functional genomic studies in V. carinata.

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DATA ACCESSIBILITY

All sequence information was uploaded to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (accession no. PRJNA167588); primer sequences were uploaded to GenBank, and accession numbers are provided in Table 1.
TABLE 4. Heterologous amplification of the 16 isolated loci of *Vriesea carinata* in five individuals of *V. reitzii*.

| Loci    | Vriesea reitzii 1 | Vriesea reitzii 2 | Vriesea reitzii 3 | Vriesea reitzii 4 | Vriesea reitzii 5 | Range in V. reitzii (bp) |
|---------|-------------------|-------------------|-------------------|-------------------|-------------------|--------------------------|
| Vcar_10 | w w w w + + + +   | + + + + + + + +   | 250–274           |                    |                    |                          |
| Vcar_31 | + + + + + + + +   | w w w w w w w w   | 220–230           |                    |                    |                          |
| Vcar_36 | + + + + + + + +   | + + + + + + + +   | 262–265           |                    |                    |                          |
| Vcar_72 | + + + + + + + +   | w w w w w w w w   | 228–251           |                    |                    |                          |
| Vcar_91 | — — — — — — — —   | + + + + + + + +   | 240–249           |                    |                    |                          |
| Vcar_93 | w w w w w w w w   | w w w w w w w w   | 246–251           |                    |                    |                          |
| Vcar_95.1 | + + + + + + + +   | w w w w w w w w   | 239–250           |                    |                    |                          |
| Vcar_115 | + + + + + + + +   | w w w w w w w w   | 200–220           |                    |                    |                          |
| Vcar_139 | + + + + + + + +   | + + + + + + + +   | 252–255           |                    |                    |                          |
| Vcar_143 | + + + + + + + +   | w w w w w w w w   | 249–255           |                    |                    |                          |
| Vcar_153 | + + + + + + + +   | w w w w w w w w   | 234–240           |                    |                    |                          |
| Vcar_258 | + + + + + + + +   | + + + + + + + +   | 231–237           |                    |                    |                          |
| Vcar_280.1 | + + + + + + + +   | + + + + + + + +   | 220–226           |                    |                    |                          |
| Vcar_293 | + + + + + + + +   | + + + + + + + +   | 256–260           |                    |                    |                          |
| Vcar_395 | w + + + + + + + + | w w w w w w w w   | 350               |                    |                    |                          |
| Vcar_501 | + + + + + + + +   | + + + + + + + +   | 350–400           |                    |                    |                          |

Note: + = successful amplification; — = unsuccessful amplification; w = weak amplification.

*Respective size ranges. Cross-amplification tests were performed using 2% agarose gel and a 50-bp ladder.

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### APPENDIX 1. Locality and voucher information for the species used in this study.

| Species | Locality | N  | Geographic coordinates | Voucher no. |
|---------|----------|----|------------------------|-------------|
| Aechmea calyculata (E. Morren) Baker | Eight Waterfalls Park, São Francisco de Paula, RS, Brazil | 1 | 29°26′S, 50°33′W | ICN 165253 |
| Aechmea caudata Lindm. | Florianópolis, SC, Brazil | 1 | 27°26′S, 48°24′W | ICN 187561 |
| Aechmea comata (Gaudich.) Baker | Florianópolis, SC, Brazil | 1 | 26°26′S, 48°31′W | ICN 184897 |
| Aechmea kertesziae Reitz | Laguna, SC, Brazil | 1 | 28°30′S, 48°45′W | ICN 167498 |
| Alcantarea extensa (L. B. Sm.) J. R. Grant | Biological Station of Santa Lúcia, Santa Tereza, ES, Brazil | 1 | 40°53′S, 19°97′W | MBML 25417 |
| Bromelia anticantha Bertol. | Mafra, SC, Brazil | 1 | 26°06′S, 59°10′W | HBR 4067 |
| Neoregelia laevis (Mez) L. B. Sm. | Graciosa highway, Morretes, PR, Brazil | 1 | 25°47′S, 56°05′W | ICN 191373 |
| Dyckia divaricata Leme & Büneker | Antônio João, MS, Brazil | 1 | 22°07′S, 48°31′W | ICN 190907 |
| Dyckia excelsa Leme | Bodoquena, MS, Brazil | 1 | 20°47′S, 56°37′W | ICN 191373 |
| Dyckia grandidentata P. J. Braun & Esteves | São Gabriel do Oeste, MS, Brazil | 1 | 19°18′S, 54°48′W | ICN 191373 |
| Dyckia leptostachya Baker | Porto Alegre, RS, Brazil | 1 | 30°07′S, 51°14′W | ICN 187141 |
| Dyckia poitii Leme | Corquinho, MS, Brazil | 1 | 19°43′S, 54°54′W | ICN 187134 |
| Vriesea altodaserrae L. B. Sm. | Rio Manso, Joinville, SC, Brazil | 1 | 26°28′S, 49°14′W | FURB 0147 |
| Vriesea carinata Wawra | Bertioga, SP, Brazil | 11 | 23°51′S, 46°08′W | ICN 177670 |
| | Graciosa Mountain, Morretes, PR, Brazil | 21 | 25°28′S, 45°50′W | ICN 177669 |
| | Ubatuba, SP, Brazil | 13 | 23°26′S, 45°50′W | RB00265965 |
| | Parque Estadual da Serra do Mar, Núcleo Santa | 24 | 23°20′S, 45°09′W | ESA08165 |
| | | Virginia, São Luiz do Paraitinga, SP, Brazil | |
| Vriesea philippocoburgii Wawra | Morretes, PR, Brazil | 1 | 25°28′S, 48°50′W | MBM3325 |
| Vriesea reitzii Leme & Costa | São Francisco de Paula, RS, Brazil | 5 | 29°44′S, 50°58′W | ICN 190912 |

Note: ES = Espirito Santo; MS = Mato Grosso do Sul; N = number of individuals sampled; PR = Paraná; RS = Rio Grande do Sul; SC = Santa Catarina; SP = São Paulo.

*Herbarium acronyms are per Index Herbariorum (Thiers, 2018).*