PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF 47 NOVEL MICROSATELITE MARKERS FOR *VELLOZIA SQUAMATA* (VELLOZIACEAE)¹

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· *Premise of the study:* We developed and validated microsatellite primers for *Vellozia squamata* (Velloziaceae), an endemic species of the cerrado (Brazilian savannas), to investigate the influence of different fire regimes on its genetic diversity and population structure.

· *Methods and Results:* Using a selective hybridization method, we tested 51 SSR loci using a natural population of *V. squamata* and obtained 47 amplifiable loci. Among these, 26 loci were polymorphic and the average values of genetic diversity were: average number of alleles per locus ($\bar{A}$) = 6.54, average number of alleles per polymorphic locus ($\bar{A}_p$) = 7.13, average observed heterozygosity ($H_o$) = 0.22, average expected heterozygosity ($H_e$) = 0.49, and average fixation index ($F_{is}$) = 0.55.

· *Conclusions:* These 26 loci allowed us to assess the effects of distinct fire regimes on the genetic structure of *V. squamata* populations with the aim of establishing strategies for the conservation of this endemic species. The markers can also be useful for future pharmaceutical studies, as the species has great potential for medicinal and cosmetic applications.

**Key words:** canela-de-ema; cerrado; fire regime; genetic diversity; *Vellozia squamata*; Velloziaceae.

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*Vellozia squamata* Pohl (= *Vellozia flavicans* Mart. ex Schult. f.; Velloziaceae) is endemic to and widely distributed throughout the cerrado of Brazil. In addition to being preferred by cattle as fodder, especially in the dry season, the species has a number of uses by local communities. The stem is used in crafts and the fibers are used for making ropes or sacks (Almeida et al., 1998), but perhaps the most promising use of the species is related to its therapeutic and cosmetic properties. For centuries it has been used as an anti-inflammatory and antirheumatic medication (Almeida et al., 1998; Brandão et al., 2012), and recent scientific studies have supported its medicinal properties (Lima, 2013). Due to its expressive antioxidant qualities and the presence of phenolic compounds, the species has potential applications for pharmaceutical and cosmetic products (Quintão et al., 2013).

*Vellozia squamata* is a self-incompatible species that exhibits morphological and physiological traits that allow it to survive the frequent fires that characterize the cerrado (Oliveira et al., 1991). In genetic terms, frequent fires are expected to increase interpopulation diversity and reduce genetic diversity within populations in fire-prone ecosystems (Premoli and Steinke, 2008; Schrey et al., 2011). Because fire regimes (intensity, frequency, and season) in the cerrado have been greatly altered by humans for agricultural and livestock breeding purposes, it is important to verify whether these novel fire regimes have genetic consequences on cerrado plants to find an adequate fire management system to conserve the biological diversity of plant populations. The implications of fire regimes on the structure and genetic diversity of fire-prone species are virtually unknown, and in Brazil, no studies on this subject are available. Therefore, we aimed to develop and validate microsatellite markers, or simple sequence repeats (SSRs), to assess the effects of distinct fire regimes on the genetic structure of *V. squamata* populations to establish strategies for the conservation of this important species.

**METHODS AND RESULTS**

Genomic DNA was extracted from lyophilized leaves (Doyle and Doyle, 1990, modified in Silva, 2013) of 48 individuals of *V. squamata* randomly taken from open cerrado (campo-sujo) habitat at the Reserva Ecológica do Instituto Brasileiro de Geografia e Estatística (RECOR-IBGE; 15° 55′–58′ S, 47° 52′–55’ W) (Appendix 1). Genomic libraries were developed following the methods in Billotte et al. (1999), as modified in Silva (2013). DNA from these individuals was digested with the restriction enzyme *AfaI* (Invitrogen, Carlsbad, California, USA), and the fragments were linked to *AfaI* (5′-CTCTTGGCTTACGCGTGGACTA-3′) adapters. The linked fragments were PCR-amplified and selected by biotin-labeled, streptavidin-associated magnetic beads with the probes (TTC)$_{10}$ (CG)$_{10}$, and (GT)$_{10}$. These fragments were PCR-amplified using the primer *AfaI* and cloned into pGEM-T vectors (Promega Corporation, Madison, Wisconsin, USA) that were subsequently transformed into competent XL1-Blue competent cells (Agilent Technologies, Santa Clara, California, USA).

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| Locus | Primer sequences (5′–3′) | Repeat motif | Allele size (bp) | Tm (°C) | GenBank accession no. |
|-------|--------------------------|--------------|----------------|---------|----------------------|
| Vsq2  | F: CTCATCTCTCTTCGGGTTGCT | (GA)₁₁⋯(CA)₆(CG)₃ | 158             | 58.0    | KC990044             |
|       | R: AGATCCTCCGCTACATGCT  |              |                 |         |                      |
| Vsq3  | F: TGATGGAGGGAAGGGAGAGTG | (GT)₉⋯(CA)₉ | 181             | 60.0    | KC990045             |
|       | R: GGCGCTGCTAAGTTATGGG  |              |                 |         |                      |
| Vsq4  | F: CAATAGTGAGTGGTTGAAGG | (TG)₁₄      | 180             | 54.0    | KC990046             |
|       | R: TGGGGTTGGGCAATAATGTA |              |                 |         |                      |
| Vsq5  | F: GCCGTGCTATCTGCAAATCT | (CA)₉(CA)₆  | 204             | 60.0    | KC990047             |
|       | R: TGATCTTAAGGCAACGACAG  |              |                 |         |                      |
| Vsq6  | F: GCACACTGCTGCTCACCTC  | (CA)₉       | 243             | 62.0    | KC990048             |
|       | R: GTATACCTCTTACCGGAAA   |              |                 |         |                      |
| Vsq7  | F: AGGCAACTAAGTGGCTTCTT | (TG)₇       | 169             | 60.0    | KC990049             |
|       | R: GGGGTTTGGGCAATGGGTTG |              |                 |         |                      |
| Vsq8  | F: GTGGTTGGAGGGAAGGGTGTG | (GT)₉⋯(CA)₁₅ | 206             | 58.0    | KC990050             |
|       | R: TGATCTTGGGTAAGCACCAA  |              |                 |         |                      |
| Vsq9  | F: GAGCTGGAATAGGGGAAAGA | (CA)₁₂      | 217             | 56.0    | KC990051             |
|       | R: GCAAATGAAAGAACCTTGA  |              |                 |         |                      |
| Vsq10 | F: GCCATGTAATAGGCGGAAA  | (TA)₉       | 120             | 62.0    | KC990051             |
|       | R: GAGGACCAACTCCCCATGAT  |              |                 |         |                      |
| Vsq11 | F: ATCATCACACACGCTCTCTT  | (AC)₁₁⋯AT(CA)₉(CG)₉ | 225    | 60.0    | KC990052             |
|       | R: CATCTATCCTCCCAACCA    |              |                 |         |                      |
| Vsq12 | F: TGGGGAGGAGTAGTGGACA   | (CA)₉⋯(TG)₇ | 187             | 62.0    | KC990052             |
|       | R: GGTCTGCTGGTTTGAGACCA  |              |                 |         |                      |
| Vsq13 | F: GCGTGACATTGGCTTCTCA  | (AC)₇       | 248             | 62.0    | KC990053             |
|       | R: AGTTTTGAGGCAACCCATTCA |              |                 |         |                      |
| Vsq14 | F: TGTCATGAGGGAAGGTTCTCA | (TG)₉⋯(TG)₇⋯(TA)₉(TG)(AG)₁₀ | 220 | 62.0    | KC990054             |
|       | R: TGAGACACGTGTTACTGCCTCA |              |                 |         |                      |
| Vsq15 | F: TAATCACAAAGCCGGTTGG  | (CA)₉⋯(CA)₉⋯(AC)₉⋯(AC)₂ | 291  | 60.0    | KC990055             |
|       | R: GAGAAGGCGATGGTGGAT  |              |                 |         |                      |
| Vsq16 | F: TTCCTAGTGTGTTGCCAGATG | (AC)₁₆     | 299             | 62.0    | KC990056             |
|       | R: GGCGTACAGGTTCCACA    |              |                 |         |                      |
| Vsq17 | F: GTTGATGAGGGAAGGAGGAGA | (TTG)₉     | 267             | 58.0    | KC990057             |
|       | R: TTGAACACACGATTTCCTACG |              |                 |         |                      |
| Vsq18 | F: CAACGAGCACCAGCTACAC | (CA)₉       | 184             | 62.0    | KC990058             |
|       | R: GGGATCTCTGGTATTGCAC  |              |                 |         |                      |
| Vsq19 | F: GAAAGGTGGAAGCCTTGA   | (AC)₉       | 222             | 56.0    | KC990059             |
|       | R: TGAAACCGCAAAATACCTC  |              |                 |         |                      |
| Vsq20 | F: GAGATGTTTGGTTGAGTG  | (GT)₉       | 250             | 60.0    | KC990060             |
|       | R: GAGGAGGAAGAATGGAATGAG |              |                 |         |                      |
| Vsq21 | F: AGACTGCGGAGAAATAACGG | (GT)₉⋯(CA)₉ | 215             | 58.0    | KC990061             |
|       | R: AAGGAAATGAGGTAGGTTG  |              |                 |         |                      |
| Vsq22 | F: AAAGGAGGTCTTGGAGGAGA | (CA)₉       | 281             | 62.0    | KC990064             |
|       | R: GGCTAGTGGTTGAGGACCC   |              |                 |         |                      |
| Vsq23 | F: TGGGCTTTGAGAATGAGTG | (TG)₉⋯(AC)₉ | 202             | 60.0    | KC990063             |
|       | R: GTTAGAATGCGGAAATATGC |              |                 |         |                      |
| Vsq24 | F: AGAATGCGGAGAAATCAGG  | (CA)₉       | 281             | 62.0    | KC990064             |
|       | R: GTGGTCTGCCAATTCTCATG  |              |                 |         |                      |
| Vsq25 | F: GCCAACGTTAGGTTAGTGGG  | (AC)₈       | 196             | 62.0    | KC990065             |
|       | R: GTGGTCTGCCAATTCTCATG  |              |                 |         |                      |
| Vsq26 | F: GTGGTACGTTAGGTTGAGTG | (AC)₁₀     | 164             | 60.0    | KC990066             |
|       | R: CTCTCCCCCTCTCCTCCTCC |              |                 |         |                      |
| Vsq27 | F: CAACGAGCACCAGCTACAC | (CA)₁₂     | 222             | 56.0    | KC990067             |
|       | R: ATGGTCTGCGCAAGATGCC  |              |                 |         |                      |
| Vsq28 | F: CACGGTATAGGCACTTCCG  | (CA)₉       | 181             | 58.0    | KC990068             |
|       | R: TTTGAAAGGAGGAGGAGGAC  |              |                 |         |                      |
| Vsq29 | F: TGGTGTCTCTGTTGCTCC   | (GA)₉       | 247             | 60.0    | KC990069             |
|       | R: TCTTGCTCTGTTGCTCC    |              |                 |         |                      |
| Vsq30 | F: GATGATGTTTGGTCTGTTGG | (AC)₁₀⋯(TG)₉ | 251             | 64.0    | KC990070             |
|       | R: GGTGATGTTTGGTCTGTTGG |              |                 |         |                      |
| Vsq31 | F: GAGATGATGTTGTTGTTG  | (CA)₁₁      | 147             | 60.0    | KC990071             |
|       | R: GCTTGATGTTGCTGTTGG   |              |                 |         |                      |
| Vsq32 | F: AAGGTGAGGAGAATGAGGAGA | (CA)₁₁   | 276             | 60.0    | KC990072             |
|       | R: GCATTTGAGTGGTTGAGGAG  |              |                 |         |                      |
| Vsq33 | F: TGATGATGAGTGGTTGCTCCTG | (AG)₉⋯ATG(CA)₉ | 223 | 58.0    | KC990073             |
|       | R: TTAACCGGCAATCAATAAGC  |              |                 |         |                      |
| Vsq34 | F: AATGATGCGTCTTCTCTGCTG | (CT)₁₃    | 146             | 58.0    | KC990074             |
|       | R: TCAACCGCATATCTTCTGCTG |              |                 |         |                      |
| Vsq35 | F: GAGGACCACTCTCCTCTCTT  | (CA)₁₂     | 217             | 58.0    | KC990075             |

TABLE 1. Characteristics of all successfully amplified SSR loci developed for *Vellozia squamata*. 

http://www.bioone.org/loi/apps
Sequencing reactions were performed using universal T7 and SP6 primers and Big Dye Terminator (version 3.1; Applied Biosystems, Foster City, California, USA). Primers flanking the identified SSR regions were designed with the software Primer3 (Rozen and Skaletsky, 1999) using the following parameters: primer size = 150–250 bp, primer melting temperature \( (T_m) = 54–66°C \), primer GC content = 40–60%, and all other parameters set at their defaults (Table 1). SSR amplification was optimized and validated for 48 individuals using fluorescently labeled M13 (5′-TAATACGACTCACTATAGGG-3′) forward primers.

PCR was performed in a 20-μL total reaction volume containing 1.0 μL of DNA (10 ng/μL), 0.32 μL of forward primer (10 μM), 0.4 μL of reverse primer (10 μM), 0.6 μL of fluorochrome-labeled primer (10 μM), 1.0 μL of dNTP mix (2.5 mM), 2.0 μL of 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, \( \text{pH} = 8.9 \)), 0.4 μL of bovine serum albumin (BSA) (2.5 μM). Thermo Fisher Scientific, Rockford, Illinois, USA), 1.6 μL of MgCl\(_2\) (25 mM), 1.2 units of Taq DNA polymerase, and ultra-pure water. The PCR program consisted of an initial denaturation step at 94°C for 5 min followed by 30 cycles of amplification (94°C for 40 s, 58°C for 40 s at the specific annealing temperature of each primer, and 72°C for 1 min), and a final elongation step at 72°C for 10 min. The amplification products were separated under denaturing conditions on a 5% (v/v) polyacrylamide gel containing 8% of urea and 1× TBE (0.045 M Tris-borate and 1.0 mM EDTA) in a LI-COR 4300S DNA Analysis System (LI-COR Biosciences, Lincoln, Nebraska, USA) for approximately 1.2 h at 70 W.

Genotyping was performed with the software SAGA (LI-COR Biosciences). We tested 51 loci in the 48 individuals collected, and amplification was unsuccessful for four loci (Vsq1, Vsq40, Vsq41, and Vsq46). For successfully amplified loci, we calculated the following variables: size polymorphism (in base pairs), average number of alleles per locus \( (A) \), expected heterozygosity \( (H_e) \), observed heterozygosity \( (H_o) \), fixation index \( (F) \), and linkage disequilibrium \( (LD) \) among loci using GENEPOP 4.2 (Raymond and Rousset, 1995) and the R package HERFSTAT (Goudet, 2005). Adherence to Hardy–Weinberg equilibrium (HWE) was tested using the Markov chain method in the software GENEPOP 4.2, with Bonferroni correction (at \( \alpha = 0.05 \)).

Of the 47 successfully amplified loci (Table 1), 26 were polymorphic (Table 2). Six loci (Vsq4, Vsq11, Vsq31, Vsq33, Vsq42, and Vsq45) had 10 or more alleles per locus. Among the 26 polymorphic loci, average values for measures of genetic diversity were as follows: average number of alleles per locus \( (A) \) = 6.54, average number of alleles per polymorphic locus \( (A_p) \) = 7.13, expected heterozygosity \( (H_e) \) = 0.49, average observed heterozygosity \( (H_o) \) = 0.32, and average fixation index \( (F) \) = 0.55. The highest expected heterozygosity was obtained for Vsq11 \( (H_e = 0.86; H_o = 0.58) \), whereas the highest observed heterozygosity was for Vsq33 \( (H_o = 0.80; H_e = 0.75) \). Most likely due to high levels of endogamy in the population, 20 polymorphic loci showed deviation from HWE after Bonferroni correction (Table 2), except: Vsq5 \( (p = 0.02) \), Vsq11 (\( p = 0.06 \)), Vsq15 (\( p = 1.00 \)), Vsq16 (\( p = 0.74 \)), Vsq23 (\( p = 0.07 \)), and Vsq36 (\( p = 1.00 \)). We found LD for four pairs of loci, perhaps caused by genetic drift and/or genetic structure. Our results showed a quite high level of inbreeding, as indicated by the average fixation index. Because this species is described by Oliveira et al. (1991) as self-incompatible, the high level of inbreeding probably results from crosses among spatially close and highly related individuals.

**CONCLUSIONS**

These are the first SSR markers developed for *V. squamata*. These loci will allow us to investigate the effects of distinct fire regimes on the genetic structure of *V. squamata* populations, which will in turn aid in the adequate management of this important species that is endemic to the Brazilian cerrado. These markers may also be instrumental for further ecological and phytotherapeutic research.

**LITERATURE CITED**

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TABLE 2. Genetic diversity values for 48 individuals of *Vellozia squamata* across 47 SSR loci.

| Locus  | A       | $H_e$    | $H_o$    | F    |
|--------|---------|----------|----------|------|
| Vsq2*  | 6       | 0.208333 | 0.687500 | 0.699200 |
| Vsq3   | 21      | 0.312500 | 0.853728 | 0.636411 |
| Vsq5   | 3       | 0.297872 | 0.513155 | 0.422162 |
| Vsq6   | 1       | 0.000000 | 0.000000 | 0.000000 |
| Vsq7*  | 3       | 0.020833 | 0.383991 | 0.946268 |
| Vsq8   | 1       | 0.000000 | 0.000000 | 0.000000 |
| Vsq9   | 1       | 0.000000 | 0.000000 | 0.000000 |
| Vsq10  | 1       | 0.000000 | 0.000000 | 0.000000 |
| Vsq11  | 14      | 0.583333 | 0.866009 | 0.326743 |
| Vsq12* | 2       | 0.000000 | 0.000000 | 0.000000 |
| Vsq13* | 2       | 0.000000 | 0.000000 | 0.000000 |
| Vsq14* | 3       | 0.083333 | 0.569518 | 0.854994 |
| Vsq15  | 2       | 0.020833 | 0.020833 | 0.000000 |
| Vsq16  | 6       | 0.125000 | 0.159211 | 0.216667 |
| Vsq17* | 4       | 0.270833 | 0.619737 | 0.565588 |
| Vsq18* | 8       | 0.416667 | 0.516228 | 0.194516 |
| Vsq19  | 1       | 0.000000 | 0.000000 | 0.000000 |
| Vsq20  | 1       | 0.000000 | 0.000000 | 0.000000 |
| Vsq21  | 1       | 0.000000 | 0.000000 | 0.000000 |
| Vsq22  | 1       | 0.000000 | 0.000000 | 0.000000 |
| Vsq23  | 1       | 0.000000 | 0.000000 | 0.000000 |
| Vsq24  | 1       | 0.000000 | 0.000000 | 0.000000 |
| Vsq25  | 1       | 0.000000 | 0.000000 | 0.000000 |
| Vsq26  | 1       | 0.000000 | 0.000000 | 0.000000 |
| Vsq27* | 5       | 0.187500 | 0.374561 | 0.502060 |
| Vsq28  | 1       | 0.000000 | 0.000000 | 0.000000 |
| Vsq29* | 7       | 0.083333 | 0.506360 | 0.836876 |
| Vsq30  | 1       | 0.000000 | 0.000000 | 0.000000 |
| Vsq31* | 10      | 0.333333 | 0.716447 | 0.537337 |
| Vsq32* | 4       | 0.208333 | 0.193640 | 0.893424 |
| Vsq33  | 11      | 0.750000 | 0.804605 | 0.068538 |
| Vsq34* | 6       | 0.395833 | 0.712719 | 0.447230 |
| Vsq35  | 1       | 0.000000 | 0.000000 | 0.000000 |
| Vsq36  | 2       | 0.145833 | 0.136623 | 0.06818 |
| Vsq37  | 1       | 0.000000 | 0.000000 | 0.000000 |
| Vsq38* | 5       | 0.208333 | 0.461623 | 0.551313 |
| Vsq39* | 5       | 0.041667 | 0.460746 | 0.910434 |
| Vsq42* | 14      | 0.541667 | 0.855044 | 0.368965 |
| Vsq43  | 1       | 0.000000 | 0.000000 | 0.000000 |
| Vsq44* | 2       | 0.000000 | 0.188596 | 1.000000 |
| Vsq45* | 11      | 0.208333 | 0.850439 | 0.756980 |
| Vsq47* | 5       | 0.229167 | 0.416886 | 0.452910 |
| Vsq48* | 9       | 0.270833 | 0.683777 | 0.609085 |
| Vsq49  | 1       | 0.000000 | 0.000000 | 0.000000 |
| Vsq50  | 1       | 0.000000 | 0.000000 | 0.000000 |
| Vsq51  | 1       | 0.000000 | 0.000000 | 0.000000 |

Note: A = total number of alleles per locus; F = estimates of fixation indices; $H_e$ = expected heterozygosity; $H_o$ = observed heterozygosity.

* Departs significantly from Hardy–Weinberg equilibrium after Bonferroni correction ($\alpha = 0.002$).

Appendix 1. Voucher and location information of samples of *Vellozia squamata* used in this study. All vouchers were collected at the Reserva Ecológica do Instituto Brasileiro de Geografia e Estatística (RECOR-IBGE).

*Vellozia squamata* Pohl—RB 287304 (deposited at the Jardim Botânico do Rio de Janeiro herbarium); Reserva Ecológica do IBGE, Distrito Federal, Brazil (−15.9597222, −47.8763889 [WGS84]); UB 38444 (deposited at the Universidade de Brasília herbarium); Reserva Ecológica do IBGE, Distrito Federal, Brazil (−15.7797, −47.9297 [WGS84]).