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Applications in Plant Sciences

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Primer Note

Development and characterization of 47 novel microsatellite 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 ($\bar{H}_o$) = 0.22, average expected heterozygosity ($\bar{H}_e$) = 0.49, and average fixation index ($\bar{F}$) = 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.

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 *Afa*I (Invitrogen, Carlsbad, California, USA), and the fragments were linked to *Afa*21 (5′-CTCTTGCTTACGCGTGGACTA-3′) and *Afa*25 (5′-CTCTTGCTTACGCGTGGACTA-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 *Afa*21 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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TABLE 1. Characteristics of all successfully amplified SSR loci developed for *Vellozia squamata*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	T_m (°C)	GenBank accession no.
Vsq2	F: CTTCATCTCCTCTGGGTGCT R: AAGATTCCGCCTCAGTGCT	$(GA)_{11}…(CA)_7(GA)_3$	158	58.0	KC990044
Vsq3	F: TGAGTGGAAGGGGGAATAGT R: TGGGGCTTGGAATAGTATGG	$(GT)_8…(CA)_5$	181	60.0	KC990045
Vsq4	F: CAAATGAGTGAGTTGGAAAGG R: TTGGGGTTGGCAAAATGTA	$(TG)_{14}$	180	54.0	KC990046
Vsq5	F: GCCGCTGCTACTTCAAAACT R: TGATCTAAATGCCAACGACAG	$(CA)_9(CT)_9$	204	60.0	KC990047
Vsq6	F: GCCAACTACCGTGCTCATC R: TGTATCTTCCTAGCCGAATCTT	$(CA)_9$	243	62.0	KC990048
Vsq7	F: AGGCAAATGGACTTGGACTT R: GGGTTTTGAGAAGGGTGTTG	$(TG)_7$	169	60.0	KC990049
Vsq8	F: TGTTTTGTTGAGAGGGTGTTG R: TGCATGTGTGTTTGAGACCA	$(GT)_5…(CA)_{15}$	206	58.0	KC990050
Vsq9	F: GACGGTGGAATACGGAGAAA R: GCAAATGAATGGAACTTGGA	$(CA)_{12}$	217	56.0	KC990051
Vsq10	F: CCGATGAATAGTGCCGAAA R: GAGGACCGAATCCCCTAAGT	$(TA)_6$	120	62.0	KC990051
Vsq11	F: ATCATCCACACGCTCCTCTT R: CATCTATCCTCCCCAAACCA	$(AC)_{11}ATA(CA)_{10}(ACA)_9$	225	60.0	KC990052
Vsq12	F: TGGGGAGGATAGATGTAGACAA R: GGTCTCAATCATGCAAAATACC	$(CA)_5…(TG)_7$	187	62.0	KC990052
Vsq13	F: GCGTGGACTATCCCTACTCA R: AGTTTAGATGCCAGACCATCAT	$(AC)_7$	248	62.0	KC990053
Vsq14	F: TGGCTATGAAGGTTTTCACAA R: TGGAGAACTGTTACTTTGCTCA	$(TG)_8…(TG)_3…(TA)_5TGT(AG)_{10}$	220	62.0	KC990054
Vsq15	F: TAATCACAAAGCACGGTTGG R: GAAGAGGAGCGTGTGGATG	$(CA)_4…(CA)_7…(AC)_3…(AC)_2$	291	60.0	KC990055
Vsq16	F: TCTTCAGTTTGTTCCAGGATG R: GCCGCTAGAGTTTCACAACC	$(AC)_{16}$	299	62.0	KC990056
Vsq17	F: TGTTGATGAAGGCAAGGAAG R: TTGAACCACGATTTCTCAGC	$(TTG)_5$	267	58.0	KC990057
Vsq18	F: CAAGCAGCACCTAGACACAC R: GGGATTCTTGGCTATTCACTG	$(CA)_7$	184	62.0	KC990058
Vsq19	F: GAAAGGTGGAGCAACTGAGC R: TGAAACCGCCAAAATCATC	$(AC)_8$	222	54.0	KC990059
Vsq20	F: GCGATGTTGTTTGTGATGG R: GGGAGAGGAAATGAAATGAAG	$(GT)_9$	250	60.0	KC990060
Vsq21	F: AGAATGCGGAGAAATCAAGG R: AAGGCAAATGGATGAGGTTG	$(GT)_6…(CA)_{10}$	215	58.0	KC990061
Vsq22	F: AATGGAGCCTTTGAGAGGAG R: CGATGTTATTTGTGATGGAACC	$(CA)_3TATACACCAC(CA)_7$	283	62.0	KC990062
Vsq23	F: TGGGGCTTGGAGTAGTATGG R: GGTAGAATGCGGAGAAATCG	$(TG)_8…(AC)_8$	202	60.0	KC990063
Vsq24	F: AGAAGTGGGAGCCTTTGTGC R: GTGTTTCGGCACTATTCATCG	$(CA)_8$	281	62.0	KC990064
Vsq25	F: GGCTAAGGCATTTGGATTGG R: TGGAAGGGTGAATAGTGTTGG	$(AC)_8$	196	62.0	KC990065
Vsq26	F: GTTGATGGTATTCGGGTTCG R: CTCTTCCCCCTTCCTTTCC	$(AC)_{10}$	164	60.0	KC990066
Vsq27	F: GAATGTCCTGCCAGAGTCC R: ATGGCTCCCAAACTTTTCC	$(CA)_{12}$	222	56.0	KC990067
Vsq28	F: CACGGATATAGGCATTCTCG R: TTTTGAAAGCGAGGGATAGC	$(CA)_6$	181	58.0	KC990068
Vsq29	F: TTGCTTGGCTCTGTACTTCC R: TCTTGACTTCGGTTTACATGC	$(GA)_6$	247	60.0	KC990069
Vsq30	F: GATCATGTTTCTTCGGTTTGG R: GGATCATTGACTCTCTCAAAGC	$(AC)_{10}…(TG)_6$	251	64.0	KC990070
Vsq31	F: AGAGGAGTGGTGTGTTGTGG R: GACTTGAACTTGGAACATTGG	$(CA)_{11}$	147	60.0	KC990071
Vsq32	F: AGTCGTCTGGATTGTTGACC R: TCCCCCATTAGATACTGTGC	$(CA)_{11}$	276	60.0	KC990072
Vsq33	F: TGGTATGCGTCTTTTATGTGG R: TTACGGACCCATCAATAAGC	$(AG)_6ATG(CA)_{13}$	223	58.0	KC990073
Vsq34	F: AATGATCCGACCTTATTCACC R: TCAACCCACGATCTTTTGG	$(CT)_{15}$	146	56.0	KC990074
Vsq35	F: TGTTGCCAATACGACATTCC R: GACAACAAGTTCCCTTTTGC	$(CA)_6$	217	58.0	KC990075

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TABLE 1. Continued.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	T_m (°C)	GenBank accession no.
Vsq36	F: ATAACCGGCATTGAGATCG R: CGGACAACCTCATCACTACC	$(GT)_6$	145	62.0	KC990076
Vsq37	F: TGGTTTGTGGTTTGTTTTGG R: GGAATCGCAAATTGAGTGG	$(TG)_{10}$	285	56.0	KC990077
Vsq38	F: CGGGAAGTCCTAAGCAACC R: TTCAGAGAGAGAGCGTTTGG	$(GA)_{11}…(CT)_5$	195	60.0	KC990078
Vsq39	F: TCCTATGTGGGGATTATTTGC R: GGACTAGCCTTCAAGTATGACG	$(AC)_7$	255	66.0	KC990079
Vsq42	F: CGATAGTGCAGCCAATGC R: GATTTTCGGGGAAGTTGG	$(CT)_6…(AC)_{18}$	300	54.0	KC990081
Vsq43	F: TATTTTGAAAGCGAGGGATAGC R: CTCGATACTCACGGATATAGGC	$(GT)_6$	192	66.0	KC990082
Vsq44	F: TGGGGCTTGGAATAGTATGG R: GTAGAATGCGGGAGAAATCG	$(TG)_5…(AC)_9$	204	60.0	KC990083
Vsq45	F: ACCTCGTCAACAGTGAGACC R: CTTCTTCAACCGCAACTCC	$(GT)_9(GA)_{16}$	229	58.0	KC990084
Vsq47	F: ATCATGCGTTCAAAAGTTGG R: AGCCTGGAAACAGATGACC	$(AC)_{11}$	207	58.0	KC990086
Vsq48	F: AGCAATTTAGTGATGGAGTTGG R: CGATGAAACAGGAACAATAAGG	$(CA)_{14}$	216	62.0	KC990087
Vsq49	F: CGAAGAAATGGTGGAAGAGG R: GTGGAACTTTGGACTTGAGC	$(CA)_{10}$	177	60.0	KC990088
Vsq50	F: CGGACAAATCTAGGAAGTGG R: GCCAAAGCTCTCAATAATGC	$(TG)_{11}$	186	58.0	KC990089
Vsq51	F: GATGGTGGTGTGAGTTGTGG R: AACAAAGGAAGCCAAAAGAGC	$(CA)_7$	178	60.0	KC990090

Note: T_m = melting temperature.

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′-CACGACGTTGTAAAACGAC-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, 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 nM), 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 pair, 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 M 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 HIERFSTAT (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 $\alpha = 0.002$).

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 ($\bar{A}$) = 6.54, average number of alleles per polymorphic locus ($\bar{A}_p$) = 7.13, average expected heterozygosity ($\bar{H}_e$) = 0.49, average observed heterozygosity ($\bar{H}_o$) = 0.22, and average fixation index ($\bar{F}$) = 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_e = 0.80; H_o = 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.

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

Locus	*A*	H_o	H_e	*F*
Vsq2*	6	0.208333	0.687500	0.699200
Vsq3	1	0.000000	0.000000	0.000000
Vsq4*	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	0.946286
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	0.328743
Vsq12*	2	0.000000	0.041228	1.000000
Vsq13*	2	0.000000	0.041228	1.000000
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	0.537373
Vsq32*	4	0.020833	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	0.688377	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 ($\alpha = 0.002$).

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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 (−15.9458333, −47.8763889 [WGS84]); RB 289062 (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]).

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