Development of Microsatellites for *Verbenoxylum reitzii* (Verbenaceae), a Tree Endemic to the Brazilian Atlantic Forest

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Primer Note

Development of microsatellites for *Verbenoxylum reitzii* (Verbenaceae), a tree endemic to the Brazilian Atlantic Forest[1]

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- *Premise of the study:* Microsatellite markers were developed for *Verbenoxylum reitzii* (Verbenaceae), a tree endemic to the Brazilian Atlantic Forest, to investigate their usefulness in population genetic studies. The loci were tested for cross-amplification in the related genera *Recordia* and *Duranta*.
- *Methods and Results:* Eleven polymorphic microsatellite markers were isolated from an enriched library of *V. reitzii* and characterized. The primers were tested on 60 individuals from three populations of this species. The number of alleles per locus ranged from two to 11, and the observed and expected heterozygosities varied from 0.0 to 1.0 and from 0.088 to 0.758, respectively. Ten loci successfully amplified in *R. boliviana* and all failed in *D. vestita*.
- *Conclusions:* Our results suggest the usefulness of the microsatellite loci developed here to access genetic variability for phylogeographic and population genetic studies in *V. reitzii*, which are important for the conservation of this rare species.

Key words: cross-amplification; microsatellite; *Recordia*; Verbenaceae; *Verbenoxylum*; *Verbenoxylum reitzii*.

Verbenoxylum Tronc. (Verbenaceae) is a monotypic genus with a restricted distribution that is endemic to the Atlantic Forest in the Brazilian states of Rio Grande do Sul and Santa Catarina. *Verbenoxylum reitzii* (Moldenke) Tronc. is a tree listed as vulnerable to extinction in the list of threatened species of Rio Grande do Sul State, Brazil (Secretaria de Estado do Meio Ambiente, 2003). It is distributed in remaining preserved fragments and riverine areas of the forest, occurring only in the lowland subtropical seasonal deciduous forest, below 550 m elevation (Troncoso, 1974). We developed and characterized 11 microsatellite loci for *V. reitzii* and tested for cross-amplification in the related and also monotypic genus *Recordia* Moldenke, a Bolivian endemic, and in a representative of the genus *Duranta* L. endemic to southeastern Brazil and Argentina (Troncoso, 1974; Sobral et al., 2006; Thode et al., in press). These are the first microsatellite loci to be developed for *V. reitzii*. The new markers presented here will be used in further genetic diversity studies, which are important to the conservation of this rare species and to preserve the Brazilian Atlantic Forest.

METHODS AND RESULTS

Total genomic DNA was extracted from silica gel–dried tissue from one individual using a cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). An enriched library methodology was used to isolate simple sequence repeats (SSRs) (Beheregaray et al., 2004). The total DNA was digested with the restriction enzyme *Rsa*I, and the fragments were linked to two oligo adapters and amplified using PCR in a thermocycler (Applied Biosystems, Foster City, California, USA) with an initial denaturation at 95°C for 4 min, followed by 20 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min, and a final extension cycle at 72°C for 8 min. The products were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), enriched in three motifs [$(dAT)_8$, $(dGA)_8$, $(dGAA)_8$], and selectively captured using streptavidin magnetic particles (Invitrogen, Carlsbad, California, USA). The selected DNA fragments were amplified by PCR with an initial denaturation at 95°C for 1 min, followed by 25 cycles of 94°C for 40 s, 60°C for 1 min, and 72°C for 2 min, and a final extension cycle at 72°C for 5 min. The resulting fragments were cloned into a pGEM-T vector (Promega Corporation, Madison, Wisconsin, USA), inserted into competent XL1-Blue *E. coli*, and incubated. A total of 100 randomly chosen clones were PCR-amplified with an initial denaturation at 95°C for 4 min, followed by 30 cycles of 94°C for 30 s, 52°C for 45 s, and 72°C for 1 min, and a final extension cycle at 72°C for 8 min. The products were purified and sequenced on a MegaBACE 1000 automated sequencer (GE Healthcare Biosciences, Pittsburgh, Pennsylvania, USA). Twenty clones presented SSRs and 14 were adequate for primer design using Primer3 version 4.0.0 (Rozen and Skaletsky, 2000), with primer sizes between 18 and 25 bp, GC contents ranging from 48% to 60%, and annealing temperatures varying from 55°C to 65°C.

The resulting markers were tested in three populations of *V. reitzii* in southeastern Brazil: Osório, Rio Grande do Sul (29°52′54″S, 50°17′15″W; $N = 20$;

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TABLE 1. Characteristics of the 13 microsatellite loci that were successfully amplified in *Verbenoxylum reitzii*.

Locus	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	T_a (°C)	GenBank accession no.
Vx01	F: TCACTTGATATGTTGCCACTTT R: TGAATGCGCTTATGGCTATC	$(CT)_7$	253–269	64	KC417432
Vx02	F: CACTTGTCGGGAGGAATGTC R: TGAGAACGCAAAACTGGATG	$(CT)_{10}$	253–277	64	KC417433
Vx03	F: TAACAGGCAATGACGGATCG R: CCCACAAACACCCGAAAG	$(CT)_9$	191–195	62	KC417434
Vx04	F: CTGTGATTGATTGGGCAGGAAG R: TTTTTGGCCCGATGGAAGTTA	$(GA)_8(GAAA)_1(GA)_8$	164–194	62	KC417435
Vx05	F: TCACATTAAATAGATGGTCAGACG R: GGTGAAACCCTATCCAATTCCAGGC	$(TG)_7(AG)_8$	153–163	62	KC417436
Vx06	F: CTGGGGCTAAAGTTGGTCAC R: TTCGCCATTTCACATCGTC	$(CT)_9$	189–217	62	KC417437
Vx07	F: CGCAAGATTCCCAATTTCTG R: TCGATTTCCACCTCGTGTG	$(CT)_9$	174–188	64	KC417438
Vx08	F: GGGGAACTTGGATGAGGAAG R: AATCTCTCCAGCACCCACTG	$(CTTT)_4(CTT)_5$	151	64	KC544260
Vx09	F: TCGGAGGTTCCATATCCTTC R: GAGTTTGTCATCAGCGACTCC	$(TTC)_{10}$	219–234	65	KC417439
Vx10	F: GACCTTGTGCGAAAATGAGC R: GTGATCTCCCTTCGCTTCC	$(GAA)_{14}$	252–276	65	KC417440
Vx12	F: GGCTATTTCGTCATTAGGCATC R: ATTCGGTAACTCACACCTGCTG	$(GA)_7$	247	64	KC544261
Vx13	F: CACAAACATGCTACGCTTGAC R: GAGGTCCTTCACTCGTCTCTTAC	$(GA)_{18}(GGGA)_1(GA)_3$	263–271	53	KC417441
Vx14	F: GCAAGACACATGCCGTTTTACC R: CTGGGCTCCTTCTCTTAACG	$(GAA)_8$	254–257	65	KC417442

Note: T_a = annealing temperature.

voucher: ICN 166749); Praia Grande, Santa Catarina (29°10′58″S, 49°59′43″W; N = 20; voucher: ICN 166755); and Orleans, Santa Catarina (28°22′16″S, 49°14′34″W; N = 20; voucher: ICN 166763). The vouchers are deposited in the Herbarium of the Universidade Federal do Rio Grande do Sul (ICN), Brazil. The DNA from 60 individuals was extracted using a CTAB protocol (Doyle and Doyle, 1987). PCR was performed in 15-µL reactions containing 10–30 ng/µL of template genomic DNA, 200 µM each dNTP (Invitrogen), 2.0 pmol each of fluorescently labeled M13(–21) primer and reverse primer, 0.4 pmol of forward primer with a 5′-M13(–21) tail, 2.0 mM $MgCl_2$ (Invitrogen), 0.5 U of *Taq* Platinum DNA polymerase, and 1× *Taq* Platinum reaction buffer (Invitrogen), with the following conditions: an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 15 s, 53–65°C for 30 s, and 72°C for 1 min, and a final extension cycle at 72°C for 10 min. The forward primers were FAM-, NED-, or HEX-labeled. The products were analyzed on MegaBACE 1000 automated sequencer using ET-ROX 550 size ladder (GE Healthcare Biosciences). Alleles were verified using Genetic Profiler version 2.0 (GE Healthcare Biosciences).

Thirteen of the 14 primers amplified successfully; of these, two were monomorphic and 11 were polymorphic. The primer sequences, repeat motif, fragment size range (bp), and the respective annealing temperatures of 13 microsatellite loci that were successfully amplified are shown in Table 1. The number of alleles per locus, observed heterozygosity (H_o), expected heterozygosity (H_e), and Hardy–Weinberg equilibrium (HWE) were analyzed with Arlequin version 3.5 (Excoffier and Lischer, 2010) (Table 2), and linkage disequilibrium using Bonferroni correction was tested with FSTAT version 1.2 (Goudet, 1995).

TABLE 2. Genetic diversity of the three populations of *Verbenoxylum reitzii*.[a]

	Osório			Praia Grande			Orleans			
Locus	*A*	H_o	H_e	A	H_o	H_e	*A*	H_o	H_e	Total *A*
Vx01	5	0.285	0.265	4	0.250	0.427	4	0.200	0.352	8
Vx02	4	0.411	0.613	4	0.600	0.541	5	0.523	0.629	7
Vx03	3	0.304	0.478	2	0.600	0.492	2	0.318	0.510	3
Vx04	7	0.608	0.672	7	0.222	0.730*	7	0.400	0.758*	11
Vx05	2	1.000	0.512*	5	0.555	0.622*	2	1.000	0.521*	5
Vx06	2	0.000	0.459*	2	0.133	0.496	2	0.000	0.507*	2
Vx07	2	0.090	0.088	4	0.411	0.572*	1	M	M	4
Vx09	2	0.318	0.273	4	0.400	0.352	3	0.217	0.300	4
Vx10	4	0.333	0.556*	6	0.578	0.603	4	0.523	0.602	7
Vx13	3	0.000	0.553*	3	0.150	0.524*	3	0.000	0.317*	4
Vx14	2	0.043	0.124	2	0.000	0.097	1	M	M	2

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; M = monomorphic.

[a] Population locality and voucher information: Osório, Rio Grande do Sul (29°52′54″S, 50°17′15″W; N = 20; voucher: ICN 166749); Praia Grande, Santa Catarina (29°10′58″S, 49°59′43″W; N = 20; voucher: ICN 166755); and Orleans, Santa Catarina (28°22′16″S, 49°14′34″W; N = 20; voucher: ICN 166763). All vouchers are deposited in the Herbarium of the Universidade Federal do Rio Grande do Sul (ICN), Brazil.

* Deviation from Hardy–Weinberg equilibrium ($P < 0.005$).

All the individuals presented one or two alleles (consistent with a diploid condition) with the expected sizes. In the Osório population, the number of alleles per locus varied from two to seven, and the H_o and H_e per locus ranged from 0.0 to 1.0 and from 0.088 to 0.672, respectively (Table 2). In the Praia Grande population, the number of alleles per locus varied from two to seven, and the H_o and H_e per locus ranged from 0.0 to 0.6 and from 0.097 to 0.73, respectively (Table 2). In the Orleans population, the loci Vx07 and Vx14 were monomorphic, the number of alleles per locus for the polymorphic loci varied from two to seven, and the H_o and H_e per locus ranged from 0.0 to 1.0 and from 0.3 to 0.758, respectively (Table 2). Considering all the populations together, the total number of alleles per locus ranged from two (Vx06 and Vx14) to 11 (Vx04), and the H_o and H_e per locus ranged from 0.0 to 1.0 and from 0.088 to 0.758, respectively (Table 2). The following loci deviated significantly ($P < 0.005$) from HWE: Vx05 and Vx06 deviated in all three populations; Vx10 and Vx13 deviated in the Osório and Praia Grande populations; and Vx02 and Vx04 deviated in the Orleans population (Table 2). No linkage disequilibrium was detected for any loci ($P > 0.004$). Cross-amplification of the 11 microsatellite loci was tested in two individuals of *R. boliviana* Moldenke, and two of *D. vestita* Cham. All markers, except Vx14, amplified successfully in *R. boliviana* with the same PCR conditions, and all failed in *D. vestita*.

CONCLUSIONS

The 11 microsatellite loci presented here revealed polymorphism in *V. reitzii* populations, providing a powerful tool for future population genetic and phylogeographic studies. The success in the cross-amplification of most of these markers in *R. boliviana* suggests that they are also useful in this species. These loci may help to provide valuable genetic information to build conservation strategies for these rare trees.

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