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ORIGINAL ARTICLE

Development and cross-validation of microsatellite markers for *Rauvolfia weddeliana* Müll.Arg. (Apocynaceae) species complex

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Abstract

Rauvolfia weddeliana is an endemic species restricted to plateau landscapes in South American savannahs. Rapid loss of habitat and expansion of agriculture in Central-West Brazil have critically reduced the original extent of savannahs, representing a major threat to its biotic diversity. Due to the discontinuous distribution of *R. weddeliana* and the vulnerability of its habitats, it is crucial to estimate the genetic diversity of remaining populations. The application of microsatellite markers is a useful approach with relative low cost and high informative potential for studies related to conservation genetics and population genetics. The development of specific libraries for endangered species may aid future studies about the connectivity of populations, reproductive biology, and genetic diversity. We developed microsatellite markers for *R. weddeliana* and tested the transferability of the markers to a closely related species, *R. gracilis*. Ten microsatellite markers were identified, and a set of primers for their amplification is presented. Most identified motifs were dimers, with lengths from 18 to 74 base pairs. Nine markers presented high informative potential (PIC > 0.5). The set of markers developed in this study will support further investigations in population genetics of *R. weddeliana* and possibility of closely related species.

Keywords "Cerrado" · Conservation genetics · Population genetics · SSR

1 Introduction

Rauvolfia L. is a genus of the Apocynaceae family widely distributed in tropical regions, with ca. 60 species, being 20 of them recorded for Brazil (Koch et al. 2015). Among the Brazilian species, *R. weddeliana* Müll.Arg. has the wider non-continuous distribution within the Brazilian savannahs (i.e., "Cerrado"), occurring frequently on sandy patches, often associated with plateaus in Central-West region (Rao 1956; Koch et al. 2007). Its geographical distribution is recorded from Chapada dos Guimarães to the Paraná Basin, reaching northeastern Paraguay in isolated patches. *Rauvolfia weddeliana* forms a taxonomic complex with two other Brazilian species that are very similar in terms of ecology and/or morphology: *Rauvolfia anomala* Rapini & I. Koch, which co-occurs in Chapada dos Guimarães and *Rauvolfia gracilis* I.Koch & Kin.-Gouv., restricted to Chapada dos Parecis, in northwestern Brazil state of Rondônia (Koch et al. 2007).

Although "Cerrado" constitutes the second largest domain in South America (Pennington et al. 2009), it is

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very vulnerable in terms of conservation, being pointed out as a conservation hot spot, mostly due to its high levels of endemism and exceptional loss of habitat (Myers et al. 2000). Originally presenting almost 2,000,000 km^2 of extension, it is estimated that more than 80% of the original coverage of "Cerrado" was lost due to human activity, and less than 7% of the remaining vegetation can be considered legally protected (Myers et al. 2000; Silva and Bates 2002). Most of *R. weddeliana* distribution is placed within Mato Grosso and Mato Grosso do Sul, core regions of extensive agronomic activity in Brazil. Although *R. weddeliana* is not listed as an endangered species (The IUCN Red List of Threatened Species. Version 2016-3. www.iucnredlist.org), this scenario of habitat vulnerability makes the assessment of its remaining genetic diversity a fundamental evaluation of the actual risk it is facing, since most of its remaining area of distribution can be degraded or even disappear in a very close future. Moreover, only a few populations of *R. weddeliana* have been registered outside conservation units in the last decade, placing as urgent an estimation of how well preserved these remaining populations are in terms of genetic diversity and connectivity.

Microsatellite markers (or Simple Sequence Repeats—'SSRs') are among the most traditional approaches in demographic studies, being used for exploring several aspects of genetic diversity with great informative potential (e.g., Zalapa et al. 2012; Hodel et al. 2016). Microsatellites are short (1–6 bp), tandemly repeated DNA sequences motifs found randomly throughout the genome of all eukaryotes (Hamada et al. 1982). Since SSR markers can provide important insights about population structure, gene flow, and genetic diversity, they have become a major trend in studies focused on genetic characterization of natural populations, genome mapping, and parentage analysis (e.g., Hodel et al. 2016). Microsatellite markers are valuable tools for phylogeographic and population genetic studies, especially due to their co-dominant inheritance and relative low cost for genotyping, when compared to SNP markers, for example.

The major drawback in the usage of microsatellite markers used to be the development of libraries, which involves several steps and substantial laboratory effort (Squirrell et al. 2003). However, recent advances in sequencing technologies and improvements on methodology have allowed for a more accessible use of these markers in non-model species (Zalapa et al. 2012). Because of its high informative power and relative cost efficiency, microsatellite markers became very popular among studies in population genetics and are currently a major choice in the field (e.g., Hodel et al. 2016). Advantages involved in adopting the use of SSRs include their abundance, reproducibility, co-dominant heritage, multi-allelic nature, and high coverage of the genome (Powell et al. 1996; Kalia et al. 2011; Hodel et al. 2016). Microsatellites are usually transferable between closely related species. In fact, more than 70% of microsatellite markers designed for eudicot species are amplified positively in sister species (Barbará et al. 2007). Of these, 10% were also transferrable to closely related genera (Barbará et al. 2007). Therefore, microsatellites have a potential application in multispecies studies regardless limitations due to high interspecific polymorphism.

Despite their popularity, only a few studies involving microsatellite markers have been developed for the tribe Vinceae from the Rauvolfioid grade of the Apocynaceae, mostly directed to *Catharanthus roseus* (L.) G.Don (Shokeen et al. 2007). So far, there has been no account of any similar study with other groups of the Rauvolfioid grade. In the present study, we fill in this gap. We developed and applied a set of SSR markers for *R. weddeliana* and estimated diversity-related parameters, as a first step to characterize the genetic diversity and structure of populations within this species.

2 Materials and methods

Population sampling and DNA extraction – We sampled an individual of *R. weddeliana* in Rondonópolis, Mato Grosso (12°31′31″S/60°23′09″W), collecting young leaves in silica, which were later stored under 4 °C. We deposited voucher material for the sample in the herbarium UEC (BRAZIL, Mato Grosso: Rondonópolis, *18 km a norte do município, nas margens da BR 364*, *sentido* Jaciara, 12°31′31″S, 60°23′09″W, 16-IX-2015, *Vidal, J.D.* 197639 (UEC) "[BRAZIL, Mato Grosso State: Rondonópolis (city), 18 km north from the city, along route BR 364, Jaciara (city) bound, 12°31′31″S, 60°23′09″W, 16-IX-2015, Vidal, J.D. 197639 (UEC)]"). We extracted total DNA with Qiagen DNeasy Plant Mini (Qiagen, Valencia, CA, USA). In addition, 70 other samples from different populations along the distribution of the species (Table 1) were genotyped to characterize the polymorphism of the recovered loci. We also genotyped a population of eight individuals of *Rauvolfia gracilis* to test for primer transferability.

Microsatellite library development – We developed an enriched library of SSR following the protocol described by Billotte et al. (1999). The first step consisted in the fragmentation of extracted DNA with the restriction enzyme *Rsa*I, in order to generate fragments of lengths between 280 and 600 bps. We then hybridized these fragments with biotinylated oligonucleotides complementary to repetitive sequences $(CT)_8$ and $(GT)_8$, aiming to select SSR rich fragments. Once hybridized, we were able to recover

Table 1 Sampled populations of *Rauvolfia weddeliana* and respective localities

Locality	Population	ID	N	Latitude	Longitude
Mato Grosso, Chapada dos Guimarães*	Faz. Chafariz	CP	14	15°17′54″S	55°50′37″W
Mato Grosso, Chapada dos Guimarães*	Véu de Noiva	VN	29	15°24′36″S	55°49′50″W
Mato Grosso, Serra de São Vicente	Jaciara	SV	5	15°49′06″S	55°20′38″W
Mato Grosso, Rondonópolis	Rondonópolis	RP	5	12°31′31″S	60°23′09″W
Goiás, PARNA das Emas*	Mineiros	CD	10	18°17′35″S	52°53′44″W
Goiás, RPPN Pousada das Araras*	Serranópolis	PA	10	18°27′03″S	52°00′22″W
Rondônia, Vilhena**	Vilhena	VL	8	18°27′03″S	52°00′22″W

Locality sampling locality. *Population* Name attributed to the population, *ID* Abbreviation of population names. *N* number of individuals sampled on population. (*): Location placed inside conservation units. (**) *Rauvolfia gracilis* population

these fragments with magnetic streptavidin-coated beads (Streptavidin MagneSphere Paramagnetic Particles, Promega, Madison, WI). To develop enriched libraries, we inserted recovered fragments into plasmid vectors and controlled their replication with x-gal and IPTG indicators. Positive clones (*i.e.*, non-recombining colonies) were submitted to an alkaline lysis in order to recover plasmidial DNA. Next, we sequenced the recovered inserts in an automatic sequencer ABI3500 XL Genetic Analyzer (Applied Biosystems, Foster City, CA, EUA) and analyzed sequences using the software Geneious 9.0 (http://www.geneious.com, Kearse et al. 2012). We trimmed low-quality ends and vector contamination from sequences using Geneious plug-in of NCBI's VecScreen (http://www.ncbi.nlm. nih.gov/VecScreen/VecScreen.html). We screened trimmed sequences for tandem repeats with Phobos plug-in (Mayer 2010). For amplification of recovered regions, we designed complementary primers to flanking regions using the software Primer3 (Rozen and Skaletsky 2000). To optimize genotyping, we adopted the following parameters for primer design: 18–22 bp primers; T_m between 45 and 65 °C, with up to 3 °C of difference between each oligo in a pair of primers; salt concentration of 50 mM; GC content between 40 and 60%; and fragments' length between 100 and 360 bp. We deposited resulting sequences and annotations in GenBank (MF447169-MF447178).

Polymerase Chain Reaction (PCR) – We amplified the selected regions through polymerase chain reaction (PCR) according to the following protocol: PCR mix was as follows: 50 mM KCl, 20 mM Tris–HCl (pH 8.4), 2 mM $MgCl_2$, 0.2 mM dNTP, 0.19 mg/mL BSA (bovine serum albumin), 0.15 mM of each primer, 1 U of *Taq* DNA polymerase, 2.5 ng template DNA. We completed final volume of reactions to 10 µL with ultrapure water. We applied the following temperature cycle: initial denaturing at 94 °C for 5 min, followed by 30 cycles of 94 °C (1 min), T_m 62 °C (1 min), 72 °C (2 min), and final elongation at 72 °C for 5 min.

Polymorphism evaluation – We amplified all ten recovered loci for 71 individuals of different populations of *R. weddeliana* and eight individuals of *R. gracillis* to estimate their polymorphism levels. Once amplified, we manually evaluated the polymorphism of these products through electrophoresis in polyacrylamide 6% gel stained with silver nitrate (Creste et al. 2001).

Also, since no information about ploidy was currently available for neither *R. weddeliana* nor *R. gracillis*, we analyzed band patterns for all loci to indirectly assess ploidy of species. We calculated basic allelic richness parameters (number of alleles, observed and expected heterozygosity) with adegenet R-package (Jombart 2008). Polymorphism information content (PIC) was calculated with R-package PopGenKit (Paquette 2012), according to the formulae proposed by Botstein et al. (1980). We also calculated F-statistics per loci (Weir and Cockerham 1984) with R-package pegas (Paradis 2010) for *R. weddeliana.* F-statistics were not calculated for *R. gracilis* due to limited sample (eight individuals from a single population). Package pegas was also applied to test deviations from Hardy–Weinberg Equilibrium (HWE). We calculated two estimates for HWE: Chi-square test based on allele frequencies and exact test with Monte Carlo permutation of alleles (1000 repetitions). We calculated the frequency estimates of null alleles (Dempster et al. 1977) with FreeNA software (Chapuis and Estoup 2007).

3 Results and discussion

We recovered a total of ten microsatellite markers from a set of 200 sequenced clones (Table 2). Motif recovery in clone amplification (5%) was higher than the average ratio in plants (2.3%; Zane et al 2002). Nine recovered loci were composed of dinucleotides and one was made of a tetra-/dinucleotide motif (SSR02). The occurrence of non-exclusively dinucleotide microsatellites is an expected product of enriched libraries since probe hybridization with

Table 2 Set of microsatellite markers recovered for *Rauvolfia weddeliana* with respective primers and properties

Locus	Sequence (5′–>3′)	% GC	T_m (°C)	H (°C)	P.size (bp)	Motif	GenBank accession
SSR01	F: ACAGGAGTGTCAAAATCCAA	40	54.8	–	250	$(GT)_9$	MF447171
	R: CTTGTTTCGAGGCAGTGATG	50	57.1	–			
SSR02	F: AGAAATGCGTATCCAATGCG	45	57	–	210	$(GTGA)_4(GA)_{19}(CA)_{10}$	MF447170
	R: AAAGATGTCAGGTCCCACTG	50	57.1	–			
SSR03	F: ATCTATTCTCCAGCCTGTGC	50	57.1	38.0	220	$(TG)_9$	MF447173
	R: GGCCCTAACAATTGGTCTCT	50	57	–			
SSR04	F: GCACACCATACACTGCTCTA	50	57.3	–	151	$(TG)_8(GT)_9$	MF447174
	R: GAGGTCAAAAAGCTGTTCCC	50	56.9	35.3			
SSR05	F: TTTCCAAAGCTGCCTCAAAG	45	56.8	–	126	$(AC)_7$	MF447169
	R: AAACATGGTTCTCACACCCT	45	56.9	35.7			
SSR06	F: GAGTGTGGAACCTGTCATGA	50	57.2	–	252	$(AC)_{15}$	MF447177
	R: GCTCCTGATGTCTGTTCAGA	50	57	42.6			
SSR07	F: AACAGCCCCTTCATCATCAA	45	57.1	–	210	$(GT)_8$	MF447175
	R: GGACAAGTTTTTCTCCCTGC	50	56.9	–			
SSR08	F: AGGCTGAAAGTAACGACTGA	45	56.5	35.5	144	$(TG)_8$	MF447172
	R: TCTGTCTCTCAGTCCCAGAA	50	57	44.0			
SSR09	F: ACCTCCGTAATTGTGGAACA	45	56.8	32.9	161	$(AC)_{13}$	MF447176
	R: CGGTTCAGGAGAGAGAAACA	50	56.9	31.0			
SSR10	F: AAAGTGCAGACTACCGACAA	45	57.1	–	286	$(GA)_{20}$	MF447178
	R: TTGAACAGTTTTTGCCGGTT	40	57	–			

Locus name attributed to the locus, *Sequence* sequence of bases of the primer (5′–>3′ sense—*F* forward, *R* reverse), *%GC* percentage of G (guanine) and C (cytosine) in the primer composition. T_m primer melting temperature, *H* primer hairpin, *P.size* size of the whole amplified fragment, *Motif* microsatellite basic unity of repetition and number of repeats

heterogeneous microsatellite regions is expected (Refseth et al. 1997). All loci were polymorphic and showed consistent amplifications following optimization. Microsatellite lengths varied from 18 to 74 base pairs, GT being the most common motif (60% of the motifs). The lengths of the amplified products ranged from 126 (SSR05) to 286 (SSR10) base pairs. Primers designed for *R. weddeliana* can be transferred between to *R. gracilis* samples, as we recovered positive cross-amplifications for all of them. All ten amplified markers were also polymorphic for both species. The number of alleles per locus ranged from five to 27, with an average of 14 and 5 alleles per locus in *R. weddeliana* and *R. gracilis*, respectively (Table 3).

Expected heterozygosity (H_E) values for each locus in *R. weddelliana* ranged from 0.52 to 0.93 (mean value = 0.76), while observed heterozygosity (H_O) ranged from 0.34 to 0.82 (mean value = 0.55, Table 3). We also observed a deficit of heterozygotes observed for most loci ($F_{IS} > 0$, except SSR04 and SSR09). On contrast, negative F_{IS} values observed for SS04 and SSR09 represent an excess of heterozygotes in comparison with the expectation under HWE. This latter pattern may be consistent with a differential survival advantage observed for heterozygotes in plants, especially for small populations (Lesica and Allendorf 1992). We identified deviation in HWE (Table 4) in locus SSR09, both for the Chi-square ($p = 0.8$) and exact tests ($p = 0.67$).

Each individual presented up to two alleles, which strongly suggests that both species are diploid, as previously reported for other *Rauvolfia* species (Carr 1978; Lewis 1980; Banerjee and Sharma 1989). Diploidy was reported for the Asian species *Rauvolfia serpentina* (L.) Benth. ex Kurz, the African species *Rauvolfia vomitoria* Afzel. (Banerjee and Sharma 1989) and the Hawaiian species *Rauvolfia sandwicensis* A.DC. (Carr 1978). Tetraploid and hexaploid species were also described for *Rauvolfia verticillata* (Lour.) Baill. and *Rauvolfia tetraphylla* L., respectively (Raghavan 1957). This study provides the first estimation of ploidy for Brazilian species of the genus.

Polymorphism information content varied from 0.22 (SSR08) to 0.83 (SSR03) (Table 3). The most informative locus (SSR02; PIC = 0.931) was also the one with largest repetition size, which is associated with higher mutation rate for microsatellites (Schug et al. 1998; Bhargava and Fuentes 2010). Higher frequencies of null alleles (29%) were observed for locus SSR05 in Serra de São Vicente population (SV) and for loci SSR06 and SSR08 in Rondonópolis population (RP). The presence of null alleles

Table 3 Allelic richness statistics for the microsatellite markers described for *Rauvolfia weddeliana* and *R. gracilis*

Locus	Total (n = 79)				*Rauvolfia weddeliana* (n = 71)				*Rauvolfia gracilis* (n = 8)		
	A	H_E	H_O	PIC	*A*	H_E	H_O	F_{IS}	*A*	H_E	H_O
SSR01	27	0.90	0.52	0.893	26	0.89	0.48	0.41	6	0.80	0.75
SSR02	27	0.94	0.74	0.931	26	0.93	0.75	0.18	10	0.88	0.75
SSR03	18	0.91	0.75	0.907	19	0.91	0.73	0.11	3	0.55	1.00
SSR04	12	0.90	0.85	0.886	12	0.89	0.82	− 0.03	4	0.73	1.00
SSR05	9	0.58	0.35	0.562	9	0.54	0.34	0.35	5	0.72	0.50
SSR06	14	0.77	0.42	0.741	14	0.80	0.45	0.25	3	0.23	0.25
SSR07	6	0.74	0.44	0.696	6	0.73	0.40	0.33	3	0.60	0.71
SSR08	5	0.49	0.42	0.462	6	0.52	0.40	0.30	2	0.38	0.50
SSR09	8	0.60	0.71	0.572	8	0.55	0.65	− 0.18	5	0.73	1.00
SSR10	10	0.81	0.43	0.788	10	0.81	0.44	0.45	8	0.79	0.50
Mean	14	0.76	0.56	0.744	14	0.76	0.55	–	5	0.64	0.70

Locus identification of the locus, *A* number of alleles observed per locus in eight samples used for characterization, H_E expected heterozygosity, H_O observed heterozygosity, F_{IS} inbreeding coefficient, *PIC* polymorphism information content

Table 4 Null allele frequencies estimated for microsatellites described for *Rauvolfia weddeliana*

Locus	Null allele frequency estimated per population						
	SV	VL	RP	CD	PA	VN	CP
SSR01	0.24	0.04	0.00	0.00	0.00	0.24	0.23
SSR02	0.11	0.07	0.00	0.04	0.00	0.10	0.01
SSR03	0.19	0.00	0.07	0.00	0.07	0.00	0.10
SSR04	0.00	0.00	0.00	0.00	0.00	0.04	0.09
SSR05	**0.29**	0.12	0.00	0.00	0.00	0.08	0.14
SSR06	0.00	0.00	**0.29**	0.00	0.00	0.07	0.06
SSR07	0.00	0.00	0.00	0.03	0.00	0.21	0.12
SSR08	0.00	0.00	**0.29**	0.00	0.00	0.00	0.17
SSR09*	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SSR10	0.27	0.16	0.20	0.01	0.16	0.19	0.10

Higher frequencies of null alleles found for the markers are depicted in bold

Locus identification of the locus, *Pr(chi²)* Chi-square test calculation for Hardy–Weinberg Equilibrium, *Pr (exact)* exact test for Hardy–Weinberg Equilibrium based on 1000 Monte Carlo permutations of alleles. *SV* population from Serra de São Vicente, Jaciara (MT), *VN* sample from Véu de Noiva, Chapada dos Guimarães (MT), *CP* sample from Cidade de Pedra (MT), *VL* population from Vilhena (RO), *RP* population from Rondonópolis (MT), *CD* population from PARNA Emas, Mineiros (GO), *PA* population from RPPN Pousada das Araras, Serranópolis (GO). *Locus with significant deviation from Hardy–Weiberg Equilibrium (Chi-square p = 0.8; exact tests p = 0.67)

may impact estimates of genetic diversity and population structure (Chakraborty et al. 1992). Due to mutations in the annealing site of primers, some alleles fail to amplify in PCR, resulting in a null allele (Chapuis and Estoup 2007). Nonetheless, it is also important to point that null alleles may also result from amplification errors, usually related to template quality (Foucault et al. 1996).

Most population parameters were consistent with a scenario of reduced gene flow. Populations of *R. weddelliana* and *R. gracilis* indeed present low densities of individuals and reduced sizes, suggesting that demographic stochasticity may play a major role in defining the structure of genetic diversity (Schaal and Leverich 1996; Honnay and Jacquemyn 2007). However, genetic diversity parameters were based on a reduced sample size and, therefore, require careful interpretation as low sample size may obscure their biological meaning. Statistics here presented are preliminary and just an illustration of the potential of the new markers. Complementary population genetic studies are being developed to elucidate key historical demographic processes within *R. weddeliana* as well to contribute with conservation policies for "Cerrado."

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Authors' contributions JDV conceived the study, sampled specimens in the field, performed laboratory procedures, analyzed data, and wrote the manuscript. MBC assisted with laboratory procedures and the elaboration of the manuscript. FMA assisted with laboratory procedures and gave a major contribution in data analysis and

discussion of results. SK assisted with the study design, laboratory procedures and the elaboration of the manuscript. APS assisted with supervision of laboratory procedures, provided methodological support and laboratory structure for the development of the libraries. IK contributed with the collection of specimens in the field and the elaboration of the manuscript.

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