New microsatellite markers for *Xerophyta dasylirioides* (Velloziaceae), an endemic species on Malagasy inselbergs

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**PREMISE:** Microsatellite markers were developed for *Xerophyta dasylirioides* (Velloziaceae), a species endemic to the Malagasy inselbergs, to explore the impact of its island-like distribution on genetic diversity and gene flow.

**METHODS AND RESULTS:** A total of 7110 perfect microsatellite loci were recovered by shotgun sequencing on an Illumina MiSeq platform. Primer pairs were designed for 40 arbitrarily selected loci. Fifteen primer pairs that generated distinct PCR products were used to genotype 80 individuals of *X. dasylirioides* from three inselberg populations. All markers were polymorphic, revealing two to 17 alleles in the overall sampling. Levels of observed and expected heterozygosity per locus ranged from zero to 1.000 and from zero to 0.850, respectively. Success rates of cross-amplification in 10 additional species of *Xerophyta* ranged from zero to 70%.

**CONCLUSIONS:** Fifteen newly developed microsatellite markers provide a toolkit for assessing population genetic parameters of *X. dasylirioides* in its unique island-like habitats.

**KEYWORDS:** desiccation-tolerance; genetic diversity; Illumina sequencing; Madagascar; rock outcrops; Velloziaceae; *Xerophyta dasylirioides*.

**PRIMER NOTE**

Inselbergs are isolated, often dome-shaped monolithic rock outcrops (either single or groups) that mainly consist of granite or gneiss (Porembski, 2007). Because inselbergs are ecologically separated from the surrounding matrix, these ecosystems are often referred to as terrestrial or sky islands (Porembski and Barthlott, 2000; Emerson, 2002). Only a few plant species possess traits that enable them to grow successfully in inselberg habitats, which are usually characterized by high temperatures, strong aridity, rocky soils, and extreme nutrient deficiency. The Velloziaceae are desiccation-tolerant vascular plants that are important floral elements on inselbergs and other rock outcrops in South America, Africa, and Madagascar (Mello-Silva et al., 2011). Studies of Velloziaceae (e.g., Loussada et al., 2013) and Bromeliaceae (e.g., Barbará et al., 2007) in South America, as well as of Gesneriaceae in Asia (e.g., Hughes et al., 2007), have indicated that rates of genetic exchange between populations located on different inselbergs can be very low even when in close proximity, thus emphasizing the potential role of isolated rock outcrops as drivers of population differentiation and ultimately speciation.

Microsatellites are informative and versatile DNA-based markers for the evaluation of intraspecific variation, population structure, and speciation (Selkoe and Toonen, 2006). Whereas microsatellite markers have been developed in two South American *Vellozia* Vand. species (Martins et al., 2012; Duarte-Barbosa et al., 2014), no such markers are yet available for Velloziaceae from the Old World. The genus *Xerophyta* Juss. (Velloziaceae) contains approximately 30 desiccation-tolerant species that are distributed from Madagascar to sub-Saharan Africa and southwestern Arabia (Behnke et al., 2013). *Xerophyta dasylirioides* Baker is an endemic species on Madagascar, where it occurs on inselbergs mainly of the Central Highlands. The delimitation of taxa within the Velloziaceae is notoriously difficult, and little is known about genetic differentiation patterns among *Xerophyta* species and populations from African and Malagasy inselbergs. We developed 15 polymorphic microsatellite markers by next-generation sequencing on an Illumina MiSeq platform to analyze the genetic diversity and population structure of *X. dasylirioides* in Madagascar and evaluate the significance of its island-like distribution in terms of speciation. We also tested the transferability
of these markers to eight other *Xerophyta* species from Madagascar (*X. pinifolia* Lam. ex Poir., *X. decaryi* Phillipson & Lowry, *X. labatii* Phillipson & Lowry, *X. setosa* Phillipson & Lowry, *X. croatii* Phillipson & Lowry, *X. isaloensis* Phillipson & Lowry, *X. lewisiae* Phillipson & Lowry, *X. tulearensis* (H. Perrier) Phillipson & Lowry) and two from continental Africa (*X. spekei* Baker, *X. retinervis* Baker; see Appendix 1).

**METHODS AND RESULTS**

Genomic DNA was extracted from lyophilized leaves of one individual plant of *X. dasylirioides* (JR1432; see Appendix 1) using a modified cetyltrimethylammonium bromide (CTAB) method (Štorchová et al., 2000). A 5-μg DNA aliquot was used for library preparation. DNA was sheared to generate fragments of on average 600-bp length, followed by adapter and barcode ligation according to Meyer and Kircher (2010). The library was size-selected by gel electrophoresis, and fragment size distribution and DNA concentration were evaluated on an Agilent BioAnalyzer High Sensitivity DNA Chip and using the Qubit DNA Assay Kit in a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Darmstadt, Germany). The final library was sequenced on an Illumina MiSeq platform (Illumina, San Diego, California, USA) generating 250-bp paired-end reads.

2.5 mM MgCl₂, 0.2 mM dNTPs, 0.05 units Taq DNA polymerase (Bioline), and 0.5 μM of each primer. Forward primers were fluorescently labeled with FAM, VIC, NED, or PET (Applied Biosystems, Foster City, California, USA; see Table 1). All loci were amplified using a touchdown PCR program with an initial denaturation at 94°C for 6 min; followed by a 12-cycle touchdown of 94°C for 45 s, 62–50°C for 30 s, and 65°C for 45 s; 18 additional cycles at 94°C for 45 s, 50°C for 30 s, and 65°C for 45 s; and a final elongation at 65°C for 10 min.

PCR products were electrophoresed on an ABI Prism 3100 sequencer (Applied Biosystems) along with a fluorescently labeled internal size standard (GeneScan 600 LIZ Size Standard; Applied Biosystems). Allele sizes were determined manually using Peak Scanner Software version 1.0 (Applied Biosystems). Numbers of alleles and levels of observed and expected heterozygosity were determined using the MISA module (Thiel et al., 2003) and considering a minimum of 10 repeat units for di-, eight for tri-, seven for tetra-, six for penta-, and five for hexanucleotide repeats, respectively, a total of 7110 perfect microsatellites were detected. For the initial screening, 40 loci were arbitrarily selected for the design of microsatellite-flanking primers using BatchPrimer3 (You et al., 2008). The criteria for primer design were (1) product size from 100 to 300 bp; (2) primer size from 18 to 23 bp; (3) annealing temperature from 50°C to 70°C; and (4) GC content of primers between 30% and 70% (Wöhrmann and Weising, 2011).

**TABLE 1.** Characteristics of 15 microsatellite markers developed for *Xerophyta dasylirioides* from Malagasy inselbergs.

| Locus  | Primer sequence (5′–3′) | Repeat motif | Fluorescent label | Allele size range (bp) | Tₓ (°C) | GenBank accession no. |
|--------|------------------------|--------------|-------------------|-----------------------|--------|-----------------------|
| Xeda_01 | F: AGTTCGGCTCGATATACCTA R: GCGAGTCTAAACAACCTTCTT  | (CTGAA)₇   | FAM               | 106–124               | 55     | MG407664              |
| Xeda_04 | F: TCGATTAGCAATATAGGATCC R: CCACAAAGGTGAATGATTTG  | (ATGTTG)₆  | NED               | 38–42                 | 55     | MH427346              |
| Xeda_12 | F: ATTTCATGACACAGGAGATTA R: TGAAGAAACACAGCTGGAG  | (TAT)₁₅    | PET               | 105–117               | 55     | MH427347              |
| Xeda_13 | F: GAAAAAGCAACACACACACGC R: GTTGTCAGGGAGTAATTAAT  | (TAT)₁₅    | VIC               | 74–107                | 55     | MG407665              |
| Xeda_15 | F: TAAAGAGTATCTCGAGAAAGAG R: TTACCGCTCTGATATTACA  | (GAA)₁₂    | NED               | 123–144               | 55     | MG407665              |
| Xeda_18 | F: TCACATCAATATACAGCTGAG R: CCTCTCTCTGCTCCTGCTT  | (TCT)₁₁    | PET               | 121–136               | 55     | MG407667              |
| Xeda_20 | F: TCTCTATACGGCCATCGATGTT R: GTGATTCAGATCTACGTGAG  | (TGA)₁₀    | VIC               | 128–146               | 55     | MH427348              |
| Xeda_23 | F: CTTACCGCTCATCAGGATGTC R: CGTATAAGAATCAGGCATCTG  | (TCC)₁₀    | FAM               | 144–159               | 55     | MG407669              |
| Xeda_25 | F: AACTCATCTCCCAATTAATTT R: TTTTTCTATCTGGGTTTTAGT  | (TCC)₁₀    | VIC               | 177–198               | 55     | MG407670              |
| Xeda_26 | F: AAGAGATGAGAAAGCGGAGGC R: GTTATGACGGAAGCGGCTCTAG  | (GAG)₁₀    | NED               | 152–167               | 54     | MG407671              |
| Xeda_28 | F: AGATGAGCAGCCGTTTACTGA R: AAAAAAAAAATGGTCTCTCTCTC  | (CGG)₁₀    | PET               | 144–188               | 55     | MG407672              |
| Xeda_31 | F: GTGACAGAGAGAGCAGACAGA R: GTGGAGCTCTCTACGATAAA  | (AG)₁₀     | FAM               | 114–156               | 55     | MH427349              |
| Xeda_34 | F: ATGCGACATTCAACATCTCC R: AGGTATGACCCCTTTCTATTG  | (CT)₁₅     | PET               | 164–212               | 55     | MH427350              |
| Xeda_39 | F: CAAGCTGTCGACTGATAAAA R: CACCTAGGCCCTTATGACCTC  | (GA)₁₃     | FAM               | 104–142               | 55     | MG407673              |

Note: Tₓ = annealing temperature.
TABLE 2. Genetic variation of 15 microsatellite loci in three natural populations of Xerophyta dasylirioides from Madagascar inselbergs.\(^*\)

| Locus | Angavokely (N = 30) | Andronovela (N = 30) | Quarry II (N = 20) |
|-------|-----------------|-----------------|-----------------|
|       | \(H_e\) | \(H_o\) | \(A\) | \(H_e\) | \(H_o\) | \(A\) | \(H_e\) | \(H_o\) | \(A\) |
| Xeda_01 | 2 | 0.700\(^b\) | 0.455 | 1 | 0.000 | 0.000 | 2 | 0.050 | 0.049 | 3 |
| Xeda_04 | 2 | 0.500 | 0.375 | 2 | 0.621\(^b\) | 0.428 | 2 | 0.950\(^b\) | 0.499 | 2 |
| Xeda_12 | 1 | 0.000 | 0.000 | 1 | 0.000 | 0.000 | 1 | 0.000 | 0.000 | 2 |
| Xeda_13 | 9 | 0.700 | 0.783 | 1 | 0.000 | 0.000 | 1 | 0.000 | 0.000 | 10 |
| Xeda_15 | 6 | 0.800 | 0.737 | 6 | 0.567 | 0.538 | 2 | 0.200 | 0.180 | 7 |
| Xeda_18 | 2 | 0.367 | 0.455 | 4 | 0.138\(^b\) | 0.628 | 2 | 0.000\(^b\) | 0.388 | 5 |
| Xeda_20 | 1 | 0.000 | 0.000 | 2 | 0.067\(^b\) | 0.358 | 3 | 0.053\(^b\) | 0.467 | 4 |
| Xeda_23 | 3 | 0.333 | 0.339 | 4 | 0.767 | 0.675 | 3 | 0.550 | 0.626 | 6 |
| Xeda_25 | 3 | 0.467 | 0.456 | 5 | 0.467 | 0.462 | 1 | 0.000 | 0.000 | 5 |
| Xeda_26 | 2 | 0.214 | 0.191 | 3 | 0.200\(^b\) | 0.331 | 2 | 0.000\(^b\) | 0.305 | 4 |
| Xeda_28 | 3 | 0.633 | 0.615 | 9 | 0.267\(^b\) | 0.518 | 1 | 0.000 | 0.000 | 5 |
| Xeda_31 | 6 | 0.739\(^b\) | 0.683 | 9 | 0.700\(^b\) | 0.850 | 6 | 0.611 | 0.765 | 17 |
| Xeda_34 | 5 | 0.379\(^b\) | 0.719 | 9 | 0.600\(^b\) | 0.841 | 2 | 0.150 | 0.219 | 14 |
| Xeda_39 | 4 | 1.000\(^b\) | 0.559 | 3 | 1.000\(^b\) | 0.589 | 2 | 1.000\(^b\) | 0.500 | 7 |
| Xeda_40 | 6 | 0.423\(^b\) | 0.541 | 9 | 0.429\(^b\) | 0.786 | 2 | 0.105\(^b\) | 0.432 | 14 |
| Mean | 4 | 0.484 | 0.461 | 4.133 | 0.388 | 0.467 | 2.133 | 0.245 | 0.295 | 7 |
| Total | 55 | – | – | 62 | – | – | 32 | – | – | 105 |

Note: – = not applicable; \(A\) = number of alleles; \(A_m\) = mean number of alleles across all 80 Xerophyta dasylirioides samples; \(H_e\) = expected heterozygosity; \(H_o\) = observed heterozygosity; \(N\) = number of individuals sampled.

\(^*\)Significant departure from Hardy–Weinberg equilibrium (chi-square, \(P < 0.05\)).

TABLE 3. Cross-amplification of primers developed in Xerophyta dasylirioides in 10 other species of Xerophyta.\(^ab\)

| Locus | XePin | XeDec | XeLab | XeSet | XeCro | Xelsa | Xelw | XeTul | XeSpe | XeRet1 | XeRet2 | XeRet3 | XeRet4 | XeRet5 | Total |
|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Xeda_01 | 112 | – | 106/112 | 112 | – | 112 | 112 | 112 | – | – | – | – | – | – | 6 |
| Xeda_04 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 0 |
| Xeda_12 | 123 | – | 105 | 105* | – | – | – | – | – | – | – | – | – | – | 4 |
| Xeda_13 | 95 | 74* | 6/2/86 | 86/98 | – | 74 | – | – | – | – | – | – | – | – | 6 |
| Xeda_15 | – | 120/123 | 120/126 | – | – | – | – | – | – | – | – | – | – | – | 2 |
| Xeda_18 | – | 130 | – | – | – | 127 | – | – | – | – | – | – | – | – | 2 |
| Xeda_20 | – | 116 | – | – | – | 110 | – | 116* | – | – | – | – | – | – | 3 |
| Xeda_23 | 137 | – | – | – | – | – | – | – | – | – | – | – | – | – | 4 |
| Xeda_25 | 153 | 141 | – | 153 | – | – | – | – | – | – | – | – | – | – | 4 |
| Xeda_26 | 183 | 201 | 183 | – | – | 189 | – | – | – | – | – | – | – | – | 5 |
| Xeda_28 | 152* | 155 | – | 152 | – | 155 | 161* | – | – | – | – | – | – | – | 5 |
| Xeda_31 | 154* | 158* | 166 | 176 | 186 | – | 168 | 154 | – | – | – | – | – | – | 7 |
| Xeda_34 | – | 138/166 | – | – | – | – | – | – | – | – | – | – | – | – | 1 |
| Xeda_39 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 0 |
| Xeda_40 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 1 |

Note: – = no amplification; * = weak amplification; Malagasy species: XePin = Xerophyta pinifolia; XeDec = X. decaryi; XeLab = X. labatii; XeSet = X. setosa; XeCro = X. croatii; Xelsa = X. lewisiae; Xelw = X. lewisii; XeTul = X. tulearensis; African species: XeSpe = X. spekei; XeRet = X. retinervis.

\(^a\)Values represent single PCR products with allele size in base pairs.

\(^b\)Voucher and locality information are provided in Appendix 1.
cross-amplifications ranged from zero to 70%, depending on the locus–species combination (Table 3). Cross-amplification in the Malagasy species was clearly more efficient than in the two African species, in which only two markers could be amplified in X. retinervis (Xeda_12 and Xeda_23). Both microsatellite loci turned out to be monomorphic across the individuals of X. retinervis tested (Table 3). The limited cross-amplification between African and Malagasy species is in accordance with expectations from the long-lasting isolation of Madagascar from continental Africa.

CONCLUSIONS

We developed 15 new nuclear microsatellite markers for the desiccation-tolerant plant X. dasylirioides, an endemic to Madagascar. The novel markers display high levels of polymorphism among 80 individual plants derived from three inselberg populations and thus provide a promising toolbox for assessing the genetic diversity and population structure of X. dasylirioides. These markers are expected to contribute to our understanding of the significance of inselbergs regarding species diversification on terrestrial islands.

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DATA ACCESSIBILITY

Raw sequencing data used for the development of microsatellite markers are available through the European Nucleotide Archive (ENA) (ERS2600133; study ID: ERP113401). Sequence information for the developed primers has been deposited to the National Center for Biotechnology Information’s GenBank, and accession numbers are provided in Table 1.

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**APPENDIX 1. Voucher and location information of samples of Xerophyta dasylirioides and related species used in the microsatellite analysis.**

| Species                        | Voucher specimen accession no.a | Collection locality / source               | Geographic coordinates                  | N  |
|-------------------------------|--------------------------------|-------------------------------------------|-----------------------------------------|----|
| X. dasylirioides Baker        | JR1432                         | Botanischer Garten Rostock                | NA                                      | 1  |
| X. dasylirioides              | JR1463–JR1492                  | Madagascar, Angavokely                    | 18°55'17"S, 47°44'19"E                 | 30 |
| X. dasylirioides              | JR1493–JR1522                  | Madagascar, Andronovelona                 | 18°38'05"S, 47°16'58"E                 | 30 |
| X. dasylirioides              | JR1523–JR1542                  | Madagascar, Quarry II                     | 18°30'44"S, 47°11'04"E                 | 20 |
| X. pinifolia Lam. ex Poir.    | P5458                          | Madagascar/IPMB Heidelberg               | NA                                      | 1  |
| X. decaryi Phillipson & Lowry | P6669                          | Madagascar, Toliara/IPMB Heidelberg       | NA                                      | 1  |
| X. labati Phillipson & Lowry  | P6671                          | Madagascar, Fianarantsoa/IPMB Heidelberg  | NA                                      | 1  |
| X. setosa Phillipson & Lowry  | P6675                          | Madagascar, Fianarantsoa/IPMB Heidelberg  | NA                                      | 1  |
| X. croati Phillipson & Lowry  | P6668                          | Madagascar, Fianarantsoa/IPMB Heidelberg  | NA                                      | 1  |
| X. isaloensis Phillipson & Lowry | P6651                         | Madagascar, Fianarantsoa/IPMB Heidelberg  | NA                                      | 1  |
| X. lewisii Phillipson & Lowry | P6652                          | Madagascar, Fianarantsoa/IPMB Heidelberg  | NA                                      | 1  |
| X. tulearensis (H. Perrier) Phillipson & Lowry | P6802 | Madagascar, Toliara/IPMB Heidelberg | NA                                      | 1  |
| X. spekei Baker               | P6425                          | Africa, Tanzania/IPMB Heidelberg          | NA                                      | 1  |
| X. retinervis Baker           | P6276, P6419, P6563, P6678, P6686 | Africa, Swaziland/IPMB Heidelberg         | NA                                      | 5  |

Note: IPMB Heidelberg = Institut für Pharmazie und Molekulare Biotechnologie der Universität Heidelberg; N = number of individuals; NA = data not available.

aVoucher deposited at the Department of Botany, University of Rostock (ROST), Rostock, Germany.