Microsatellite marker development for the tetraploid Veronica aragonensis (Plantaginaceae) using next-generation sequencing and high-resolution melting analyses

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To cite this version:

Nélida Padilla-García, Teresa Malvar-Ferreras, Josie Lambourdière, M. Montserrat Martínez-Ortega, Nathalie Machon. Microsatellite marker development for the tetraploid Veronica aragonensis (Plantaginaceae) using next-generation sequencing and high-resolution melting analyses. Applications in Plant Sciences, 2018, 6 (5), pp.e1154. 10.1002/aps3.1154. hal-01822845

HAL Id: hal-01822845
https://hal.sorbonne-universite.fr/hal-01822845
Submitted on 25 Jun 2018

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Microsatellite marker development for the tetraploid
Veronica aragonensis (Plantaginaceae) using
next-generation sequencing and high-resolution melting analyses

Nélida Padilla-García1,2,3, Teresa Malvar-Ferreras1,2, Josie Lambourdière4, M. Montserrat Martinez-Ortega1,2, and Nathalie Machon3

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PREMISE OF THE STUDY: The tetraploid Veronica aragonensis (Plantaginaceae) is a narrow endemic to the Iberian Peninsula. Specific microsatellite markers were developed to investigate genetic structure and diversity.

METHODS AND RESULTS: A total of 15 polymorphic markers were characterized on three populations of V. aragonensis, using a microsatellite-enriched library on an Ion Torrent sequencer and high-resolution melting (HRM) analyses to rapidly discard unreliable, multicopy, and/or monomorphic loci. Allele number per locus ranged from one to five, and levels of observed heterozygosity per population varied from 0.142 ± 0.301 to 0.281 ± 0.369. Most primers also amplified in the closely related species V. rosea and in three subspecies of V. tenuifolia.

CONCLUSIONS: The species-specific microsatellite markers developed here represent an essential tool to provide genetic information on the population level for V. aragonensis. The low levels of variation detected highlight the importance of continued efforts to improve conservation of the species.

KEY WORDS: high-resolution melting (HRM) analyses; microsatellites; Plantaginaceae; polyploidy; Veronica aragonensis.

Veronica aragonensis Stroh (Plantaginaceae) is a perennial herb included in the diploid–polyploid complex Veronica subsect. Pentasepalae Benth., one of the four subsections recognized within Veronica subgen. Pentasepalae M. M. Mart. Ort., Albach & M. A. Fisch. (Rojas-Andrés et al., 2015). This endemic plant is restricted to three disjunct mountain areas in the Iberian Peninsula (Martínez-Ortega et al., 2009). It is one of the few highly specialized plants growing in Iberian limestone mountain scree (between 1000 and 2300 m). Given that it is a rare species, it is included in regional catalogs and Red Lists from Spain (Cabezudo et al., 2005; Alcántara de la Fuente et al., 2007).

A set of microsatellite markers was previously developed for other species from Veronica subsect. Pentasepalae (i.e., V. austriaca L. subsp. jacquinii (Baumg.) Watzl and V. orbiculata A. Kern.; López-González et al., 2015). However, preliminary cross-transferability tests performed for most of these loci resulted either in monomorphic patterns or unsuccessful amplifications in V. aragonensis (results not shown). Successful cross-species transfer of nuclear microsatellite markers is usually limited—particularly in terms of polymorphism—by large evolutionary distances (Ellegren et al., 1995; Barbará et al., 2007). Previous studies suggested that V. aragonensis is relatively isolated from the remaining species of the subsection (Martínez-Ortega et al., 2004; Rojas-Andrés et al., 2015; Padilla-García et al., 2018). This may be precluding cross-transferability success. In this situation, new microsatellite markers must be developed to address the study of gene flow patterns and genetic structure in the narrow endemic V. aragonensis.

METHODS AND RESULTS

Genomic DNA from one individual of V. aragonensis (Appendix 1) was extracted following the cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). A DNA library was
generated on an Ion Torrent Personal Genome Machine Sequencer (Life Technologies, Saint Aubin, France) using the kit NEBNext Fast DNA Fragmentation & Library Prep Set for Ion Torrent (New England Biolabs, Ipswich, Massachusetts, USA). Then, an emulsion PCR was performed to enrich the library, and sequencing was performed using 800 flows (generating ca. 100–400 bp read lengths) on an Ion 316 v2 sequencing chip (Life Technologies). Sequences were submitted to the National Center for Biotechnology Information’s (NCBI) Sequence Read Archive (SRA; accession no. SRP129594). BioProject information and BioSample records are available under accession numbers PRJNA429875 and SAMN08362105, respectively. From a total of 737,951 sequences, 11,604 microsatellites were detected, and 4572 of them were in singleton sequences. Microsatellite selection and primer design were performed using QDD version 3.1 (Meglécz et al., 2014) for detecting unique microsatellite sequences, with a minimum of five repeats, a PCR product

<table>
<thead>
<tr>
<th>Locus</th>
<th>Repeat motif</th>
<th>Product size (bp)</th>
<th>No. of dF/dT peaks</th>
<th>$T_a$ range (K)</th>
<th>Variability</th>
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<tr>
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<td>03</td>
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<tr>
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<td>No amplification</td>
</tr>
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<td>31</td>
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<td>36</td>
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<td>46</td>
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<tr>
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<td>1</td>
<td>0.20</td>
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</tr>
<tr>
<td>48</td>
<td>(AAAC)$_{8}$</td>
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<td>0.20</td>
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<tr>
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<td>(AG)$_{10}$</td>
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</tr>
<tr>
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<td>(AT)$_{10}$</td>
<td>369</td>
<td>1</td>
<td>0.00</td>
<td>Monomorphic</td>
</tr>
</tbody>
</table>

Note: — = no data due to failed PCR amplification; dF/dT peaks = peaks observed in the melt curve when plotting the derivative of fluorescence over temperature; $K =$ melting temperature range; $T_a =$ annealing temperature. *Differences observed in curve shape among samples.
size of 90–450 bp, an optimal temperature of 60°C, and 50% of GC. Primers were designed for 1727 microsatellites, of which 50 were tested for polymorphism.

High-resolution melting (HRM) analyses were used as a previous screening to rapidly identify PCR failure, monomorphism, or multitype status of microsatellite loci (Arthofer et al., 2011). Amplification and HRM analyses were performed on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA) using SsoFast EvaGreen 2× SuperMix (Bio-Rad Laboratories) with 0.4 μM simple sequence repeat (SSR)–specific primers and 2 μL of template DNA (ca. 32 ng/μL) in a 10 μL total reaction volume. Cycling conditions were 2 min initial hot start at 98°C, followed by 40 cycles of 98°C for 5 s, 50°C for 10 s, and 72°C for 20 s. Cycling was followed by 20 s hold at 95°C to ensure a homogeneous denaturation of amplicons. HRM analysis consisted of an initial 5 s hold at 65°C and ramping from 65°C to 95°C in 0.2°C steps. Each cycle was followed by 20 s holds at 95°C to ensure a homogeneous denaturation of amplicons. HRM analysis consisted of an initial 5 s hold at 65°C and ramping from 65°C to 95°C in 0.2°C steps. Each step was held for 5 s before the fluorescence was acquired. Melting-temperature ranges and differences in curve shape among samples were analyzed as a measure of SSR size variation. Of 50 loci tested by HRM analyses, eight did not amplify in quantitative PCR and 16 were excluded as monomorphic due to the low melting temperature range observed (≤0.20 K). Although polymorphism was difficult to confirm by this methodology, it allowed us to screen for robust amplification and single-copy status of the tested loci (Table 1).

The remaining loci (26) were genotyped on 11 individuals from a single population of V. aragonensis and 10 individuals from 10 different populations (Appendix 1) to evaluate the intrapopulation and interpopulation polymorphism of the markers, respectively. PCR reactions contained 1.25 μL of Taq Pol Buffer (10×), 0.8 mM of dNTPs mix (Life Technologies, Carlsbad, California, USA), 1.5 mM of MgCl2, 0.08 μM of each forward primer modified with an M13 tail, 0.2 μM of reverse primer, 0.2 μM of fluorescent-labeled M13 universal primer, 0.5 units Taq DNA Polymerase (Biotools B&M Labs S.A., Madrid, Spain), 40–50 ng of DNA template, and H2O up to a final volume of 12.5 μL. Gradient PCRs were performed to test all primers as follows: 2 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 55.7–62.5°C, and 50 s at 72°C; followed by 10 cycles of 1 min at 94°C, 1 min at 53°C, and 50 s at 72°C; with a final extension of 15 min at 72°C. PCR products were visualized on a 2.5% agarose gel and separated on a multi-capillary sequencer ABI PRISM 3730 (Applied Biosystems, Waltham, Massachusetts, USA) using GeneScan 500 LIZ Size Standard (Applied Biosystems). Electropherograms were visualized and scored with GeneMarker version 1.8 software (SoftGenetics, State College, Pennsylvania, USA). Fifteen primer combinations (Table 2) displaying clear peak patterns and polymorphism were combined in multiplex reactions according to annealing temperature and amplicon sizes. Sequences from these loci were deposited in GenBank (Table 2).

To characterize the microsatellite loci, a total of 92 individuals from three populations representing the main distribution areas of this endemic species were used (34, 23, and 35 individuals from the Herin, Arguis, and La Sagra populations, respectively; see

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**TABLE 2. Description of 15 microsatellite loci developed in Veronica aragonensis.**

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer sequences (5′–3′)</th>
<th>Fluorescent dye</th>
<th>Repeat motif</th>
<th>Allele size range (bp)</th>
<th>Tm (°C)</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
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<td>04</td>
<td>F: TCACGTGAACTTACCTCCCATC R: AAACAAAGATTGCGAGCTCTG</td>
<td>S-FAM</td>
<td>(AGAT)ₙ</td>
<td>94–126</td>
<td>61.2</td>
<td>MF946655</td>
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<tr>
<td>10</td>
<td>F: AGCATGACCTGTTCTACAC R: CAGATGATGTCAGTCATAACC</td>
<td>S-FAM</td>
<td>(AAT)ₙ</td>
<td>115–160</td>
<td>55.7</td>
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</tr>
<tr>
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<td>(AA)ₙ</td>
<td>124–134</td>
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</tr>
<tr>
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<td>(AAAC)₂</td>
<td>105–125</td>
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<td>(AC)₉</td>
<td>127–137</td>
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</table>

Note: Tm = annealing temperature.
A new set of nuclear microsatellite loci has been developed for the tetraploid endemic species *V. aragonensis*. These markers will be useful for assessing genetic diversity and structure, as well as levels of gene flow within and among populations of this endangered endemic species. The amplification of some of these loci was successful for other closely related taxa (i.e., *V. rosea*, *V. tenuifolia* subsp. *fontqueri*, *V. tenuifolia* subsp. *javalambrensis*, and *V. tenuifolia* subsp. *tenuifolia*). Therefore, they will be suitable to provide genetic information on these additional North African and Iberian endemics.

### CONCLUSIONS

A new set of nuclear microsatellite loci has been developed for the tetraploid endemic species *V. aragonensis*. These markers will be useful for assessing genetic diversity and structure, as well as levels of gene flow within and among populations of this endangered endemic species. The amplification of some of these loci was successful for other closely related taxa (i.e., *V. rosea*, *V. tenuifolia* subsp. *fontqueri*, *V. tenuifolia* subsp. *javalambrensis*, and *V. tenuifolia* subsp. *tenuifolia*). Therefore, they will be suitable to provide genetic information on these additional North African and Iberian endemics.

### ACKNOWLEDGMENTS

The authors thank all colleagues from the Service de Systématique Moléculaire team (CNRS-MNHN) for technical support in NGS and HRM analyses: R. Debruyne, D. Gey, C. Bonillo, and B. Gangloff. We are grateful to J. Abdelkrim for assistance with primer design and analyses, and for his continuous support. This work was funded in part by the Spanish Ministerio de Economía y Competitividad (CGL2009-07555 and CGL2012-32574), the MNHN, and the Universidad de Salamanca (Ph.D. grant to N.P. cofounded by Banco Santander).

### LITERATURE CITED


Appendix 1). Three loci (27, 29, and 53) did not amplify across all 92 samples, and loci 11 and 12 resulted in imperfect microsatellites. These markers were finally discarded due to difficult scoring. For the remaining 10 loci, sample size, number of alleles, observed heterozygosity, and expected heterozygosity (with and without correction of allele dosages for polyploids) were evaluated with GENODIVE (Meirmans and Van Tienderen, 2004). The number of alleles per locus ranged from one to five. Levels of observed heterozygosity (mean ± SD) were 0.246 ± 0.273, 0.281 ± 0.369, and 0.142 ± 0.301 for the Nerín, Arguís, and La Sagra populations, respectively (Table 3).

The transferability of 15 primer pairs was tested in four closely related taxa from the Ibero–North African group recognized within subsection *Pentasepalae* (Padilla-García et al., 2018): *V. rosea* Desf., *V. tenuifolia* Asso subsp. *fontqueri* (Pau) M. M. Mart. Ort. & E. Rico, *V. tenuifolia* subsp. *javalambrensis* (Pau) Molero & J. Pujadas, and *V. tenuifolia* subsp. *tenuifolia*. Six individuals from different populations of each taxon were tested in agarose gel (Appendix 1). Five primer pairs were successfully amplified in all four taxa, whereas loci 13 and 21 failed in *V. rosea* individuals. Three loci exhibited no amplification in any of the tested samples, and five markers exhibited several bands or limited interspecific transferability (Table 4).

### TABLE 3. Genetic characterization of 10 polymorphic microsatellites in three populations of Veronica aragonensis.a

<table>
<thead>
<tr>
<th>Locus</th>
<th>Nerín (N = 34)</th>
<th>Arguís (N = 23)</th>
<th>La Sagra (N = 35)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>H₀</td>
<td>Hₑ</td>
</tr>
<tr>
<td>04</td>
<td>2</td>
<td>0.273</td>
<td>0.383</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>0.364</td>
<td>0.504</td>
</tr>
<tr>
<td>13</td>
<td>4</td>
<td>0.281</td>
<td>0.522</td>
</tr>
<tr>
<td>15</td>
<td>1</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>21</td>
<td>1</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>23</td>
<td>4</td>
<td>0.118</td>
<td>0.120</td>
</tr>
<tr>
<td>25</td>
<td>2</td>
<td>0.125</td>
<td>0.065</td>
</tr>
<tr>
<td>26</td>
<td>2</td>
<td>0.265</td>
<td>0.490</td>
</tr>
<tr>
<td>36</td>
<td>2</td>
<td>0.938</td>
<td>0.500</td>
</tr>
<tr>
<td>56</td>
<td>2</td>
<td>0.091</td>
<td>0.211</td>
</tr>
</tbody>
</table>

Note: A = number of alleles; H₀ = expected heterozygosity; Hₑ = observed heterozygosity; Nₑ = expected heterozygosity corrected by allele dosage; Hₑ-e = observed heterozygosity; N = number of individuals sampled.

aVoucher information and geographic coordinates for the populations are available in Appendix 1.

### TABLE 4. Cross-amplification tests of 15 microsatellite loci developed in Veronica aragonensis across four additional taxa.a

<table>
<thead>
<tr>
<th>Locus</th>
<th>V. rosea (N = 6)</th>
<th>V. tenuifolia subsp. fontqueri (N = 6)</th>
<th>V. tenuifolia subsp. javalambrensis (N = 6)</th>
<th>V. tenuifolia subsp. tenuifolia (N = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>04</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>10</td>
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<td>11</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>≡</td>
<td>≡</td>
<td>≡</td>
<td>≡</td>
</tr>
<tr>
<td>21</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>23</td>
<td>≡</td>
<td>≡</td>
<td>≡</td>
<td>≡</td>
</tr>
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<td>—</td>
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<td>53</td>
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<td>—</td>
</tr>
<tr>
<td>56</td>
<td>—</td>
<td>—</td>
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</tr>
</tbody>
</table>

Note: + = successful amplification; ≡ = several bands; * = weak amplification; — = no amplification; N = number of individuals tested.

aVoucher information and geographic coordinates for the populations are available in Appendix 1.
APPENDIX 1. Geographic location and voucher information for the Veronica samples used in this study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Collector no.</th>
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<th>Locality</th>
<th>Collection date</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Altitude (m)</th>
<th>Voucher code</th>
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<td>Spain. Pyrenees. Huesca, Nerín, La Estiba mountain</td>
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<td>42°35′57.00″N</td>
<td>00′00″30.70″E</td>
<td>1728</td>
<td>SALA 154410</td>
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<td><em>V. aragonensis</em></td>
<td>NPG12</td>
<td>1</td>
<td>Spain. Pyrenees. Huesca, betw. Chía and Plan, Sahún mountain pass</td>
<td>08/07/2014</td>
<td>42°33′14.40″N</td>
<td>02′26″11.00″E</td>
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<td>SALA 154268</td>
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<td><em>V. aragonensis</em></td>
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<td>09/07/2014</td>
<td>42°27′45.00″N</td>
<td>02′27″56.20″E</td>
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<td>36°28′36.10″N</td>
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<td><em>V. tetuifolia</em> Asso subsp. fontqueni (Pau) M. M. Mart. Ort. &amp; E. Rico</td>
<td>MO886</td>
<td>1</td>
<td>Spain. Granada, betw. Calar de Sta. Bábara &amp; Relumbre cliff, Sierra de la Neves</td>
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<th>Longitude</th>
<th>Altitude (m)</th>
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<td>2</td>
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Note: N = number of individuals.  
*Samples were used as follows: 1 = individual used for genomic library; 2 = individuals used for pre-screening analyses and genotyping tests; 3 = individuals used for characterization of microsatellites; 4 = individuals used for cross-amplification tests.  
BRC = Blanca M. Rojas-Andrés, collector; DP = Daniel Pinto-Carrasco, collector; MO = M. Montserrat Martínez-Ortega, collector; NLG = Noemí López-González, collector; NPG = Nélida Padilla-García, collector; VL = Víctor Lucía, collector.  
Date format is day/month/year.  
Vouchers deposited at the Universidad de Salamanca herbarium (SALA) and Universidad de Málaga herbarium (MGC).  
*No voucher is available from this population due to its conservation status (Critically Endangered).