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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 Martínez-Ortega1,2, and Nathalie Machon3

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 nonreliable, 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.
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

| Locus | Repeat motif | Product size (bp) | No. of dF/dT peaks | T_a range (K) | Variability |
|-------|-------------|------------------|---------------------|---------------|-------------|
| 01    | (AAT)_12   | 90               | —                   | —             | No amplification |
| 02    | (AT)_10    | 90               | 2                   | 1.20          | Potentially polymorphic |
| 03    | (AC)_9     | 91               | 1                   | 0.40          | Potentially polymorphic |
| 04    | (AGAT)_9   | 95               | 1                   | 0.40*         | Potentially polymorphic |
| 05    | (AAT)_9    | 97               | —                   | —             | No amplification |
| 06    | (AGAT)_9   | 103              | —                   | —             | No amplification |
| 07    | (AAGAC)_9  | 103              | 1                   | 0.40          | Potentially polymorphic |
| 08    | (AT)_9     | 105              | —                   | —             | No amplification |
| 09    | (AG)_9     | 105              | —                   | —             | No amplification |
| 10    | (AAT)_9    | 111              | 1–2                 | 1.20          | Potentially polymorphic |
| 11    | (AT)_10    | 113              | 1                   | 0.40*         | Potentially polymorphic |
| 12    | (AAAC)_10  | 114              | 1                   | 0.60          | Potentially polymorphic |
| 13    | (AC)_9     | 115              | 1                   | 0.40*         | Potentially polymorphic |
| 14    | (AAT)_9    | 126              | 1–2                 | 4.00          | Potentially polymorphic |
| 15    | (AC)_10    | 127              | 1                   | 0.20*         | Potentially polymorphic |
| 16    | (AT)_10    | 129              | —                   | —             | No amplification |
| 17    | (ATATC)_9  | 137              | 1                   | 0.80          | Potentially polymorphic |
| 18    | (ACAT)_9   | 138              | 1                   | 0.40          | Potentially polymorphic |
| 19    | (AG)_9     | 138              | 1                   | 0.20          | Monomorphic |
| 20    | (AAAT)_9   | 141              | 1                   | 0.40          | Potentially polymorphic |
| 21    | (AAAT)_6   | 150              | 2                   | 2.00          | Potentially polymorphic |
| 22    | (AAT)_10   | 156              | 1                   | 0.00          | Monomorphic |
| 23    | (AAG)_9    | 162              | 1                   | 0.40*         | Potentially polymorphic |
| 24    | (AT)_10    | 162              | —                   | —             | No amplification |
| 25    | (AG)_10    | 166              | 1                   | 0.20*         | Potentially polymorphic |
| 26    | (AAAAT)_10 | 167              | 1–2                 | 0.60          | Potentially polymorphic |
| 27    | (AAT)_9    | 180              | 1                   | 0.20*         | Potentially polymorphic |
| 28    | (AAAG)_9   | 185              | 1                   | 0.20          | Monomorphic |
| 29    | (AT)_10    | 190              | 1                   | 0.40*         | Potentially polymorphic |
| 30    | (AAAAC)_9  | 191              | 1                   | 0.20          | Monomorphic |
| 31    | (AC)_9     | 191              | 1                   | 0.40          | Potentially polymorphic |
| 32    | (AT)_9     | 195              | 1–2                 | 2.80          | Potentially polymorphic |
| 33    | (AAT)_9    | 195              | —                   | —             | No amplification |
| 34    | (AAG)_9    | 196              | 1                   | 0.40          | Potentially polymorphic |
| 35    | (AC)_9     | 198              | 1                   | 0.40          | Potentially polymorphic |
| 36    | (AT)_9     | 207              | 2                   | 0.20*         | Potentially polymorphic |
| 37    | (AAAT)_9   | 207              | 1                   | 0.20          | Monomorphic |
| 38    | (AC)_10    | 219              | 1                   | 0.20          | Monomorphic |
| 39    | (AATC)_9   | 225              | 1                   | 0.20          | Monomorphic |
| 40    | (AAC)_9    | 240              | 1                   | 0.20          | Monomorphic |
| 41    | (AC)_10    | 240              | 1                   | 0.60          | Potentially polymorphic |
| 42    | (AT)_9     | 253              | 1                   | 0.20          | Monomorphic |
| 43    | (AT)_10    | 260              | 2                   | 0.40*         | Potentially polymorphic |
| 44    | (ACT)_9    | 265              | 2                   | 0.00          | Monomorphic |
| 45    | (ACTC)_9   | 270              | 1                   | 0.20          | Monomorphic |
| 46    | (AAAG)_9   | 290              | 1                   | 0.20          | Monomorphic |
| 47    | (AT)_9     | 297              | 1                   | 0.20          | Monomorphic |
| 48    | (AAAAC)_9  | 340              | 1                   | 0.20          | Monomorphic |
| 49    | (AG)_9     | 340              | 1                   | 0.00          | Monomorphic |
| 50    | (AT)_10    | 369              | 1                   | 0.00          | Monomorphic |

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 multiplicity 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, 60°C for 10 s, and 72°C for 20 s. Cycling 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 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 Nerín, Arguís, and La Sagra populations, respectively; see Table 2). Description of 15 microsatellite loci developed in *Veronica aragonensis*.

| Locus | Primer sequences (5′–3′) | Fluorescent dye | Repeat motif | Allele size range (bp) | Tm (°C) | GenBank accession no. |
|-------|--------------------------|----------------|--------------|-----------------------|---------|----------------------|
| 04    | F: TCACTGTAACATCTACTCCCATC | S-FAM | (AGAT)₉ | 94–126 | 61.2 | MF946655 |
|       | R: AACACAAGAGTAGGCCTGCCTG | | | | | |
| 10    | F: AGCATGACTGGTTTCCATCAC | S-FAM | (AAT)₉ | 115–160 | 55.7 | MF946656 |
|       | R: CGATATCGGGTAACTCGTCTC | | | | | |
| 11    | F: CAACTGTAGAAAGAAGCTGCAAC | PET | (AAT) | 124–134 | 61.2 | MF946657 |
|       | R: CAGGAATACAGCCTGGTCCTC | | | | | |
| 12    | F: TCAARTGCCTACCATCTTCTGTCG | NED | (AAC) | 105–125 | 61.2 | MF946658 |
|       | R: CATTCAATCTGCAGTCTGGGG | | | | | |
| 13    | F: TCCATCTTGGAAAGTCATC | WC | (AC) | 127–137 | 61.2 | MF946659 |
|       | R: CATGAACAACACATTTGAGAACC | | | | | |
| 15    | F: TGATGCTAGAGTGGAGGACC | PET | (AAT) | 145–157 | 61.2 | MF946660 |
|       | R: AAAGCATAAAGAGCACTAATCCTC | | | | | |
| 21    | F: TCAGAAGTCTGTCGGCAACCT | NED | (AAAT) | 169–193 | 61.2 | MF946661 |
|       | R: CATTCTACGCTTCTTCTTAACG | | | | | |
| 23    | F: TTCTCTCCTTTCTCTGACAGCG | VIC | (AAQ) | 164–206 | 57.2 | MF946662 |
|       | R: TTTGCAACATATTTTCAAGATCCG | | | | | |
| 25    | F: TGATATTTCTTTTAAAGTACCCG | NED | (AG) | 180–206 | 57.2 | MF946663 |
|       | R: TATGCTCTGTATTGCGAAGCG | | | | | |
| 26    | F: CGGTGTCATCTGGAAATTTCCC | VIC | (AAAT) | 172–187 | 61.2 | MF946664 |
|       | R: CGTGTAAATTTGACGTTTTGTTG | | | | | |
| 27    | F: TGCTGATGGTCGTAATTCGAC | S-FAM | (AAT) | 167–225 | 61.2 | MF946665 |
|       | R: AATCTGCGTATTGTTCTTG | | | | | |
| 29    | F: CAGATGACCTTGGACAGGGATC | PET | (AT) | 205–225 | 61.2 | MF946666 |
|       | R: TCTACTGCTTCTCTCTCTGCGC | | | | | |
| 36    | F: ACACTGCACTTTGAGAATTACCATC | AT | (AC) | 226–240 | 61.2 | MF946667 |
|       | R: ATGATGGGCTTATGAGTGGT | | | | | |
| 53    | F: GCTAAATACGACACACACACAGAGATG | NED | (AT) | 104–122 | 55.7 | MF946668 |
|       | R: TGATGCTGCTTTAATCCACC | | | | | |
| 56    | F: AAAGCTTAACTTTGAGTGGTG | VIC | (AAAG) | 128–148 | 61.2 | MF946669 |
|       | R: CCAAAGCTTTATTTCATCTAAT | | | | | |

Note: Tm = annealing temperature.
TABLE 3. Genetic characterization of 10 polymorphic microsatellites in three populations of Veronica aragonensis.a

| Locus | Nerín (N = 34) | Arguis (N = 23) | La Sagra (N = 35) |
|-------|----------------|----------------|------------------|
|       | \( H_o \) | \( H_e \) | \( H_{e-d} \) | \( H_o \) | \( H_e \) | \( H_{e-d} \) | \( H_o \) | \( H_e \) | \( H_{e-d} \) |
| 04    | 2     | 0.273 | 0.383 | 0.379 | 3     | 0.000 | 0.372 | 0.372 | 2     | 0.000 | 0.115 | 0.115 |
| 10    | 2     | 0.364 | 0.504 | 0.504 | 3     | 0.200 | 0.556 | 0.567 | 5     | 0.206 | 0.418 | 0.460 |
| 13    | 4     | 0.281 | 0.522 | 0.520 | 2     | 1.000 | 0.512 | 0.512 | 3     | 0.970 | 0.583 | 0.507 |
| 15    | 1     | 0.000 | 0.000 | 0.000 | 1     | 0.000 | 0.000 | 0.000 | 2     | 0.029 | 0.015 | 0.015 |
| 21    | 1     | 0.000 | 0.000 | 0.000 | 2     | 0.130 | 0.506 | 0.506 | 1     | 0.000 | 0.000 | 0.000 |
| 23    | 4     | 0.118 | 0.120 | 0.120 | 3     | 0.318 | 0.448 | 0.470 | 1     | 0.000 | 0.000 | 0.000 |
| 25    | 2     | 0.125 | 0.065 | 0.064 | 4     | 0.077 | 0.641 | 0.643 | 3     | 0.030 | 0.075 | 0.075 |
| 26    | 2     | 0.265 | 0.490 | 0.489 | 2     | 0.000 | 0.290 | 0.290 | 1     | 0.000 | 0.000 | 0.000 |
| 36    | 2     | 0.938 | 0.500 | 0.498 | 3     | 0.905 | 0.585 | 0.551 | 2     | 0.182 | 0.096 | 0.094 |
| 56    | 2     | 0.091 | 0.211 | 0.210 | 2     | 0.182 | 0.486 | 0.486 | 2     | 0.000 | 0.059 | 0.059 |

Total 22 0.246 ± 0.273 0.280 ± 0.222 0.278 ± 0.222 25 0.281 ± 0.369 0.440 ± 0.185 0.440 ± 0.183 22 0.142 ± 0.301 0.136 ± 0.200 0.133 ± 0.190

Note: \( N \) = number of individuals sampled; \( H_o \) = observed heterozygosity; \( H_e \) = expected heterozygosity corrected by allele dosage; \( H_{e-d} \) = expected heterozygosity; \( e-d \) = expected heterozygosity corrected by allele dosage; \( e-d \) = observed heterozygosity; \( N \) = number of individuals sampled.

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

| Locus | V. rosea (N = 6) | V. tenuifolia subsp. fontqueri (N = 6) | V. tenuifolia subsp. javalambrensis (N = 6) | V. tenuifolia subsp. tenuifolia (N = 6) |
|-------|-----------------|-------------------------------------|--------------------------------------|-----------------------------------|
| 04    | +               | +                                   | +                                    | +                                 |
| 10    | +               | +                                   | +                                    | +                                 |
| 11    | +               | +                                   | +                                    | +                                 |
| 12    | +               | +                                   | +                                    | +                                 |
| 13    | —               | —                                   | *                                    | +                                 |
| 15    | ≡               | ≡                                   | ≡                                    | ≡                                 |
| 21    | —               | +                                   | +                                    | +                                 |
| 23    | ≡               | ≡                                   | —                                    | ≡                                 |
| 25    | —               | —                                   | —                                    | —                                 |
| 26    | *               | *                                   | *                                    | *                                 |
| 27    | +               | +                                   | +                                    | +                                 |
| 29    | —               | —                                   | *                                    | +                                 |
| 36    | ≡               | ≡                                   | —                                    | —                                 |
| 53    | —               | —                                   | —                                    | —                                 |
| 56    | —               | —                                   | —                                    | —                                 |

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

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.

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### APPENDIX 1. Geographic location and voucher information for the *Veronica* samples used in this study.

| Species                  | Collector no. | N | Locality                          | Collection date | Latitude | Longitude | Altitude (m) | Voucher code |
|--------------------------|---------------|---|-----------------------------------|-----------------|----------|-----------|--------------|--------------|
| *V. aragonensis* Stroh   | NPG18          | 34| Spain. Pyrenees. Huesca, Nerín, La Estiba mountain | 25/07/2015       | 42°35′57″N 0°00′30″W | 1728      | SALA 154410 |
| *V. aragonensis*         | NPG12          | 1 | Spain. Pyrenees. Huesca, betw. Chía and Plan, Sahún mountain pass | 08/07/2014       | 42°33′14″40″N | 1722      | SALA 154268 |
| *V. aragonensis*         | NPG13          | 1 | Spain. Pyrenees. Huesca, Bisaurri, Gabás mountain | 09/09/2014       | 42°27′45″00″N | 1830      | SALA 154272 |
| *V. aragonensis*         | NPG15          | 1 | Spain. Pyrenees. Huesca, Seira, Barbarrunz. Cociella massif | 10/08/2014       | 42°30′44″08″N | 1806      | SALA 154362 |
| *V. aragonensis*         | NPG22          | 1 | Spain. Pyrenees. Huesca, Yésero, Del Puerto cliff, Tendehera mountains | 14/07/2014       | 42°40′12″10″N | 1971      | SALA 155054 |
| *V. aragonensis*         | NPG67          | 1 | Spain. Pyrenees. Huesca, Laspuña, Ceresa mountain pass to the Peña Montañesa | 27/07/2015       | 42°29′25″00″N | 1713      | SALA 121537 |
| *V. aragonensis*         | NPG68          | 1 | Spain. Pyrenees. Huesca, Vilas del Turbón, Turbón mountain | 29/07/2015       | 42°24′14″30″N | 1527      | SALA 121536 |
| *V. aragonensis*         | NPG24          | 2 | Spain. Pre-Pyrenees. Huesca, Nocito, Tozal de Guara mountain | 03/08/2015       | 42°17′13″80″N | 1980      | SALA 121538 |
| *V. aragonensis*         | NPG25          | 1 | Spain. Pre-Pyrenees. Huesca, betw. Arguis & Bentué de Ralas | 04/08/2015       | 42°19′54″10″N | 1075      | SALA 121540 |
| *V. aragonensis*         | MO2047         | 23| Spain. Pre-Pyrenees. Huesca, betw. Arguis & Bentué de Ralas | 17/07/2007       | 42°19′59″90″N | 1075      | SALA 121540 |
| *V. aragonensis*         | NPG28          | 35| Spain. Granada, Puebla de Don Facduque, La Sagra mountain | 31/07/2015       | 37°57′12″70″N | 2285      | SALA 93529  |
| *V. rosea* Desf.         | DP783          | 1 | Morocco. Ifran. Azrou, near Djebel Hebri | 07/07/2010       | 33°21′10″60″N | 1927      | SALA 149323 |
| *V. rosea*               | NLG88          | 1 | Morocco. Taroudant. Souss-Massa-Drâa, Jebel Siroua | 21/07/2013       | 30°46′38″40″N | 2611      | SALA 155071 |
| *V. rosea*               | VL173          | 1 | Morocco. Tinghir. Souss-Massa-Drâa, Ighl Mgoun | 20/07/2013       | 31°32′11″70″N | 3031      | SALA 155074 |
| *V. rosea*               | MO5502         | 1 | Algeria. Tlemcen. Ktcherbe | 15/06/2010       | 34°34′30″20″N | 1517      | SALA 149324 |
| *V. rosea*               | MO5510         | 1 | Algeria. Batna, Djebel Ichali summit | 19/06/2010       | 35°28′18″90″N | 1745      | SALA 149338 |
| *V. rosea*               | MO5518         | 1 | Algeria. Tizi Ouzou. Djurjura Natural Park, Tizi n-Kouial | 20/06/2010       | 36°28′36″10″N | 1607      | SALA 149325 |
| *V. tenuifolia* Asso subsp. fontqueri (Pau) M. M. Mart. Ort. & E. Rico | MO886 | 1 | Spain. Granada, betw. Calar de Sta. Bárbara & Relumbre cliff, Sierra de Baza | 08/06/2000       | 37°22′44″50″N | 1900      | SALA 95042  |
| *V. tenuifolia* subsp. fontqueri | MO1905 | 1 | Spain. Málaga, Ronda, Sierra de las Nieves | 05/06/2006       | 36°41′41″30″N | 1733      | MGC 46659   |
| *V. tenuifolia* subsp. fontqueri | MO1512 | 1 | Spain. Málaga, Ronda, Sierra de las Nieves | 23/05/2002       | 37°41′00″00″N | 1730      | MGC 46659   |

(continues)
| Species                          | Collector no.\(^ab\) | \(N\) | Locality                          | Collection date\(^c\) | Latitude         | Longitude         | Altitude (m) | Voucher code\(^d\) | Voucher coded \(^e\) |
|---------------------------------|----------------------|-------|-----------------------------------|------------------------|------------------|-------------------|--------------|--------------------|-------------------|
| *V. tenuifolia* subsp. *fontqueri* | MO1518*             | 1     | Spain. Almería, Abla, Sierra de Baza | 24/05/2002             | 37°22′09″N        | 2°50′18″W         | 2167         |                    | No voucher*        |
| *V. tenuifolia* subsp. *fontqueri* | MO1519\(^a\)        | 1     | Spain. Almería, Dalias, Sierra de Gádor | 25/05/2002             | 36°51′54.60″N    | 2°47′53.00″W      | 1900         | SALA 120855        |
| *V. tenuifolia* subsp. *fontqueri* | MO1520\(^a\)        | 1     | Spain. Almería, Dalias, Sierra de Gádor | 25/05/2002             | 36°52′27.00″N    | 2°47′12.60″W      | 1900         | SALA 120855        |
| *V. tenuifolia* subsp. *javalambrensis* (Pau) Molero & J. Pujadas | BR222\(^a\)        | 2     | Spain. Salamanca, La Mata de la Armuña | 20/06/2012             | 41°02′16.20″N    | 5°40′36.50″W      | 789          | SALA 149328        |
| *V. tenuifolia* subsp. *javalambrensis* | DP1322\(^a\)        | 2     | Spain. Soria, Villaciervos, El Santo | 08/06/2013             | 41°46′08.10″N    | 2°38′54.60″W      | 1228         | SALA 150477        |
| *V. tenuifolia* subsp. *javalambrensis* | NLG05\(^a\)         | 2     | Spain. Guadalajara, Atienza, Ermita de Sta. Lucía | 27/05/2013             | 41°11′23.16″N    | 2°52′43.02″W      | 1120         | SALA 155105        |
| *V. tenuifolia* subsp. *tenuifolia* | BR237\(^a\)         | 1     | Spain. Barcelona, Collsuspina, Sta. Coloma de Castellterçol | 14/06/2013             | 41°49′24.00″N    | 2°10′36.24″E      | 905          | SALA 155065        |
| *V. tenuifolia* subsp. *tenuifolia* | BR241\(^a\)         | 1     | Spain. Huesca, Arro, S. Vitorián's monastery | 17/06/2013             | 42°24′36.84″N    | 0°13′20.34″E      | 605          | SALA 155117        |
| *V. tenuifolia* subsp. *tenuifolia* | MO6059\(^a\)        | 1     | Spain. Teruel, betw. Bordón & Calanda | 10/06/2013             | 40°41′36.60″N    | 0°19′9.50″W       | 769          | SALA 155099        |
| *V. tenuifolia* subsp. *tenuifolia* | MO6068\(^a\)        | 1     | Spain. Barcelona, betw. Su & Fontelles | 16/06/2013             | 41°53′17.88″N    | 1°34′42.42″E      | 713          | SALA 155121        |
| *V. tenuifolia* subsp. *tenuifolia* | NLG09\(^a\)         | 1     | Spain. Barcelona, Montserrat | 13/06/2013             | 41°36′37.00″N    | 1°46′13.10″E      | 746          | SALA 155098        |
| *V. tenuifolia* subsp. *tenuifolia* | NLG16\(^a\)         | 1     | Spain. Barcelona, Sta. Cecilia de Voltregà, ermita de Sta. Perpetua | 15/06/2013             | 41°59′54.90″N    | 2°12′9.90″E       | 663          | SALA 155125        |

Note: \(N\) = number of individuals.

\(^a\)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.

\(^b\)BR = Blanca M. Rojas-Andrés, collector; DP = Daniel Pinto-Carraico, collector; MO = M. Montserrat Martínez-Ortega, collector; NLG = Noemí López-González, collector; NPG = Nélida Padilla-García, collector; VL = Víctor Lucía, collector.

\(^c\)Date format is day/month/year.

\(^d\)Vouchers deposited at the Universidad de Salamanca herbarium (SALA) and Universidad de Málaga herbarium (MGC).

\(^e\)No voucher is available from this population due to its conservation status (Critically Endangered).