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CHARACTERIZATION OF 12 POLYMORPHIC SSR MARKERS IN VERONICA SUBSECT. PENTASEPALAE (PLANTAGINACEAE) AND CROSS-AMPLIFICATION IN 10 OTHER SUBGENERA

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• Premise of the study: Microsatellite primers were developed in the perennial herbs of the diploid-polyploid complex Veronica subsect. Pentasepalae (Plantaginaceae) to investigate the role that hybridization has played in the evolution of the group, which includes several endangered species.

• Methods and Results: Twelve pairs of primers leading to polymorphic and readable markers were identified and optimized from V. jacquinii and V. oribilculata using a microsatellite-enriched library method and 454 GS-FLX technique. The set of primers amplified dinucleotide to pentanucleotide repeats, and the number of alleles per locus ranged from one to six, one to 11, and one to nine for V. oribilculana, V. javalambrensis, and V. rosea, respectively. Transferability analyses were performed in 20 species corresponding to 10 different subgenera.

• Conclusions: These results indicate the utility of the newly developed microsatellites across Veronica subsect. Pentasepalae, which will help in the study of gene flow patterns and genetic structure.

Key words: conservation; hybridization; Plantaginaceae; polyploid complex; Veronica subsect. Pentasepalae.

The genus Veronica L. (Plantaginaceae) comprises ca. 450 species, which are grouped into 12 subgenera with between two and 180 species each (Albach et al., 2004; Garnock-Jones et al., 2007). It includes some perennials of relative economic importance in ornamental horticulture and others that are well-known widespread weeds. Additionally, several species of Veronica are registered on the International Union for Conservation of Nature Red List (http://www.iucnredlist.org/) and other regional catalogs of endangered plants (e.g., Peñas de Giles et al., 2004), or are threatened plants with narrow distribution areas (e.g., Petrova and Vladimirov, 2009). Veronica subsect. Pentasepalae Bentham is a monophyletic diploid-polyploid complex and one of the four subsections currently recognized within the also monophyletic Veronica subgen. Pentasepalae M. M. Mart. Or., Albach & M. A. Fischer (Albach et al., 2008). This subsection comprises ca. 20 perennial taxa and is represented in the temperate regions of Eurasia with one species in North Africa. The complex seems to be of recent origin and divergence, as many diploid representatives are still extant and short branches are found in the phylogenetic analyses based on ITS and plastid DNA sequence data (Rojas-Andrés et al., 2015). Although the diploid species are characterized by subtle morphological differences, each has been recovered as monophyletic in previous studies. Hybridization and polyploidization are widespread in the group, and several authors (Lehmann, 1937; Scheerer, 1949; Rojas-Andrés et al., 2015) have concluded that gene flow and complex relationships among polyploids and their diploid relatives might exist. Interestingly, some of the diploid and polyploid species belonging to Veronica subsect. Pentasepalae are Mediterranean orophytes that face a high risk of extinction with climate warming and/or grow in Important Plant Areas (IPAs; IPA online database: http://www.plantlifeipa.org/reports.asp), regions that display exceptionally rich floras of biogeographic interest (Rojas-Andrés et al., 2015). Given that current gene flow and introgression may have blurred species limits, particularly in hybrid zones, accurate investigations of gene flow patterns within and among Veronica subsect. Pentasepalae populations are necessary for conservation and species delimitation purposes.

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METHODS AND RESULTS

Microsatellite development—For the microsatellite library, silica gel–dried leaves of 12 diploid individuals of V. jacquinii Baumg. and V. orbicalata A. Kern. were selected from eight different populations (Appendix 1). Flow cytometry was checked using flow cytometry. A microsatellite library was prepared by Genoscreen (Lille, France) using a 454 GS-FLX (Roche Diagnostics, Meylan, France) high-throughput DNA sequencer (Malausa et al., 2011). Genomic DNA was extracted using the cetantrimethylammonium bromide method described in Doyle and Doyle (1987). The DNA was fragmented and enriched with TG, TC,
## Table 1. Characterization of 12 polymorphic nuclear microsatellite loci isolated from Veronica subsect. Pentasepalae.  

| Locus | Primer sequences (5′–3′) | Fluorescent dye | Repeat motif | Allele size range (bp) | T<sub>α</sub> (°C) | GenBank accession no. |
|-------|-------------------------|----------------|-------------|-----------------------|----------------|----------------------|
| 8     | F: TGGATGTGACTGATGGTCA  | 5-FAM          | (TGA)<sub>h</sub> | 92–95                 | 55            | KR698358             |
| 10    | R: TTACCTCTCATACCTCCCC  |                | (AGT)<sub>h</sub> | 113–119               | 55            | KR698359             |
| 11    | F: GCTGAGTGGTGAAGAAGG   | PET            | (TGAT)<sub>h</sub> | 113–133               | 58            | KR698360             |
| 19    | R: CACCATATCACACGGCTGA  |                |             |                       |               |                      |
| 26    | F: ATCGTTGTCCTCATCTCTCC | NED            | (CAA)<sub>h</sub> | 87–102                | 56            | KR698363             |
| 27    | R: CACTTGTCTCACAGCTGCC  |                |             |                       |               |                      |
| 35    | F: CTATTTGAGACCATGTCGA  | NED            | (TATC)<sub>h</sub> | 106–130               | 52            | KR698365             |
| 49    | R: TGTTACGACATTTATGGTGATT| PET            | (TTGTG)<sub>h</sub> | 201–221               | 55            | KR698364             |
| 50    | F: TGGATGACGAGCTGTGATT  | VIC            | (AGA)<sub>h</sub> | 400–460               | 50            | KR698367             |
| 52    | R: ATAAAACATACATCCTGAC  | NED            | (TATC)<sub>h</sub> | 358–391               | 52            | KR698368             |
| 54    | F: CCAATTGAACTTACATACC | NED            | (ACAT)<sub>h</sub> | 283–301               | 52            | KR698369             |

Note: T<sub>α</sub> = annealing temperature.  
*All values are based on 90 samples from three Veronica populations.  
^Range of fragment sizes does not include the M13 tail (5′-GTAAACGACGCTT-3′) attached to the forward primer.

AAC, AAG, AGG, ACAT, and ACTC motifs. A total of 32,052 high-quality sequences were obtained. Analyses of these sequences with QDD software (Meglécz et al., 2010) revealed 3010 sequences with microsatellite motifs, for which 1959 pairs of primers were obtained. Given that it is too time consuming and not affordable to check all of the primer pairs obtained, 54 of them with low primer pair penalty and different lengths and repeat motifs were selected. These primers were ordered (Eurofins, Ebersberg, Germany) to evaluate polymorphic loci on 12 individuals from the complex Veronica jacquinii–V. orbiculata complex were tested in two individuals from three species, each from a different clade (V. orsiniana Ten. [core clade], V. javalambrensis Pau [Iberian clade], and V. rosea Desf. [North African clade]), using the same PCR conditions. Twelve polymorphic primer pairs were selected (see Appendix 2 for additional primers). Following the procedure developed by Schuelke (2000), the sequence-specific forward primers were marked at the 5′ end with an M13 tail (5′-GTAAAAACGACGCTT-3′) (Eurofins), which was then labeled with 5-FAM, VIC, NED, or PET fluorescent dyes (Table 1) (Life Technologies). The PCR mix contained 1× PCR Green GoTaq Buffer (Promega Corporation, Madison, Wisconsin, USA), 0.25 mM of each dNTP (Life Technologies, Carlsbad, California, USA), 0.33 mM of each primer, 0.5 units GoTaq DNA Polymerase (Promega Corporation), and 18.2 ng of DNA template. PCRs used the following conditions: an initial step at 94°C, 1 min at 50–58°C, and not affordable to check all of the primer pairs obtained, 54 of them with low primer pair penalty and different lengths and repeat motifs were selected. These primers were ordered (Eurofins, Ebersberg, Germany) to evaluate polymorphic loci on 12 individuals from the complex Veronica jacquinii–V. orbiculata complex were tested in two individuals from three species, each from a different clade (V. orsiniana Ten. [core clade], V. javalambrensis Pau [Iberian clade], and V. rosea Desf. [North African clade]), using the same PCR conditions. Twelve polymorphic primer pairs were selected (see Appendix 2 for additional primers). Following the procedure developed by Schuelke (2000), the sequence-specific forward primers were marked at the 5′ end with an M13 tail (5′-GTAAAAACGACGCTT-3′) (Eurofins), which was then labeled with 5-FAM, VIC, NED, or PET fluorescent dyes (Table 1) (Life Technologies). The PCR mix contained 1× PCR Green GoTaq (Promega Corporation), 0.2 mM of each dNTP, 0.16 mM of each reverse and fluorescent-labeled PCR primers, and 50 s of each reverse and fluorescent-labeled PCR primers, and 0.2 mM of each dNTP, 0.16 mM of each reverse and fluorescent-labeled PCR primers, and 18.2 ng of DNA template. PCRs used the following conditions: an initial step at 94°C for 2 min; followed by 35 cycles of 1 min at 94°C, 1 min at 50–58°C, and 50 s at 72°C; and a final extension of 15 min at 72°C. All the reactions were conducted on a Mastercycler pro S thermocycler (Eppendorf, Hamburg, Germany). The PCR products were separated by electrophoresis on a 2.5% agarose gel and sent to Macrogen Europe sequencing service (Amsterdam, The Netherlands).

### Table 2. Results of initial primer screening of polymorphic loci in three populations corresponding to three different taxa belonging to Veronica subsect. Pentasepalae.

| Locus | V. orsiniana (n = 30) | V. javalambrensis (n = 30) | V. rosea (n = 30) |
|-------|-----------------------|-----------------------------|-------------------|
|       | A | H<sub>A</sub> | H<sub>E</sub> | HWE<sup>b</sup> | A | H<sub>A</sub> | H<sub>E</sub> | HWE<sup>b</sup> | A | H<sub>A</sub> | H<sub>E</sub> | HWE<sup>b</sup> |
| 8     | 2 | 0.933 | 0.506 | 0.000*** | 2 | 0.167 | 0.155 | 1.000 ns | 3 | 0.033 | 0.097 | 0.017* |
| 10    | 2 | 0.000 | 0.066 | 0.017*  | 1 | 0.500 | 0.500 | 0.388 ns  | 4 | 0.233 | 0.298 | 0.968 ns  |
| 13    | 2 | 0.167 | 0.440 | 0.001*** | 6 | 0.700 | 0.697 | 0.852 ns  | 9 | 0.690 | 0.736 | 0.144 ns  |
| 19    | 2 | 0.333 | 0.488 | 0.125 ns  | 4 | 0.376 | 0.381 | 0.448 ns  | 5 | 0.690 | 0.743 | 0.391 ns  |
| 26    | 2 | 0.700 | 0.525 | 0.140 ns  | 10 | 0.433 | 0.432 | 1.000 ns  | 3 | 0.233 | 0.213 | 1.000 ns  |
| 35    | 2 | 0.400 | 0.488 | 0.447 ns  | 3 | 0.333 | 0.420 | 0.100 ns  | 4 | 0.769 | 0.669 | 0.860 ns  |
| 54    | 3 | 0.567 | 0.733 | 0.000*** | 3 | 0.367 | 0.310 | 0.632 ns  | 4 | 0.600 | 0.494 | 0.399 ns  |

Note: — = not amplified; A = number of alleles; H<sub>A</sub> = expected heterozygosity; H<sub>E</sub> = observed heterozygosity; HWE = Hardy–Weinberg equilibrium probabilities; n = number of individuals sampled.  
<sup>a</sup>See Appendix 1 for locality and voucher information for each population.  
<sup>b</sup>Deviations from HWE were not statistically significant (ns) and statistically significant at *P < 0.05, **P < 0.01, and ***P ≤ 0.001.
### TABLE 3. Amplification success of all microsatellite primers across 20 species from 10 subgenera of Veronica.

| Subgenera | Collector no. | Species | 8 | 10 | 13 | 19 | 20 | 26 | 27 | 35 | 49 | 50 | 52 | 54 |
|-----------|---------------|---------|---|----|----|----|----|----|----|----|----|----|----|----|
| Veronica subg. Beccabunga (Hill) M. M. Mart. Ort., Albach & M. A. Fisch. | DCA350 | V. gentianoides | w | s | + | w | — | — | + | — | — | — | — | — |
| Veronica subg. Beccabunga | DCA297 | V. gentianoides | s | s | + | w | — | — | + | — | — | — | — | — |
| Veronica subg. Beccabunga | MO1598 | V. gentianoides | — | — | — | | — | — | + | — | — | — | — | — |
| Veronica subg. Chamaedrys (W. D. J. Koch) M. M. Mart. Ort., Albach & M. A. Fisch. | KBch67 | V. chamaedrys subsp. chamaedryoides | s | s | w | + | + | w | + | — | — | — | — | s |
| Veronica subg. Chamaedrys | KBch54 | V. vindobonensis | s | + | w | + | s | + | — | — | — | s | — | — |
| Veronica subg. Cochlidiosperma (Rchb.) | DCA403 | V. cymbalaria | + | + | s | w | s | + | — | — | — | — | — | — |
| Veronica subg. Cochlidiosperma | KBch67 | V. cymbalaria | + | + | + | w | s | s | + | — | — | — | — | — |
| Veronica subg. Cochlidiosperma | HMM31 | V. cymbalaria | + | + | + | w | s | s | + | — | — | — | — | — |
| Veronica subg. Cochlidiosperma | HMM32 | V. cymbalaria | + | + | + | w | s | s | + | — | — | — | — | — |
| Veronica subg. Cochlidiosperma | HMM29 | V. panormitana | + | s | + | — | s | — | + | — | w | — | — | — |
| Veronica subg. Cochlidiosperma | HMM30 | V. trichadena | + | s | + | s | — | + | — | w | — | — | — | — |
| Veronica subg. Pelikosperma (E. B. J. Lehm.) | DCA434 | V. triphyllus | + | + | w | s | s | w | s | — | — | — | + | w |
| Veronica subg. Pseudolysimachium (W. D. J. Koch) M. M. Mart. Ort., Albach & M. A. Fisch. | DCA144 | V. filiformis | w | + | s | w | s | w | + | — | — | — | — | — |
| Veronica subg. Pseudolysimachium | DCA954 | V. filiformis | s | + | s | w | s | s | + | — | — | — | v | s |
| Veronica subg. Pseudolysimachium | DCA892 | V. filiformis | s | + | s | w | s | w | + | + | — | — | — | — |
| Veronica subg. Pseudolysimachium | KB847 | V. orchidea | s | + | w | s | s | + | — | — | — | — | — | s |
| Veronica subg. Pseudolysimachium | KBps54 | V. orchidea | s | + | + | — | + | + | — | — | — | — | — | — |
| Veronica subg. Pseudolysimachium | KBps57 | V. orchidea | w | s | s | w | — | + | — | — | — | — | w | — |
| Veronica subg. Pseudolysimachium | BF1726 | V. incarna | w | s | + | w | — | + | — | — | — | — | — | — |
| Veronica subg. Pseudoveronica J. B. Armstr. | PG2878 | V. speciosa | s | s | + | s | s | + | s | s | s | — | — | — |
| Veronica subg. Pseudoveronica | HMM69 | V. salicornioides | s | s | + | s | s | + | s | s | s | — | — | — |
| Veronica subg. Pseudoveronica | HMM38 | V. hectori subsp. coarctata | w | s | + | s | s | w | s | s | s | — | — | s |
| Veronica subg. Pseudoveronica | HMM39 | V. ochracea | s | s | + | s | s | s | s | s | — | — | — | s |
| Veronica subg. Pseudoveronica | HMM40 | V. planopetiolata | s | + | s | s | s | s | s | s | s | — | — | s |
| Veronica subg. Pseudoveronica | HMM37 | V. caxerontae | s | s | w | s | s | s | + | — | s | s | s | s |
| Veronica subg. Pseudoveronica | LS1408 | V. fruticans | s | s | s | + | s | s | s | s | — | w | + | — |
| Veronica subg. Stenocarpon (Boriss.) M. M. Mart. Ort., Albach & M. A. Fisch. | DCA71 | V. fruticulosa | s | + | + | s | s | + | s | — | + | + | — | — |
| Veronica subg. Stenocarpon | DCA124 | V. missurica | w | + | w | + | + | s | — | — | + | + | w | — |
| Veronica subg. Veronica | DCA114 | V. officinalis | w | w | s | w | w | w | w | w | w | w | w | w |

*Note: + = successful amplification; — = no amplification; s = several bands; w = weak amplification.*

*Abbreviations (collector numbers): BF = Bozo Frajman; DCA = Dirk C. Albach; HMM = Heidi M. Meudt; KB = Katharina E. Bardy; LS = Lena Struwe; PGJ = Phil Garnock-Jones. DNA samples are deposited at Carl von Ossietzky Universität Oldenburg (Germany).*
and 50 ng of DNA template in a total volume of 15 μL. Conditions of the PCR amplification were as described above, adding 10 cycles of 1 min at 94°C, 1 min at 53°C, and 50 s at 72°C before the final extension. PCR products were analyzed with GeneMarker AFLP/Genotyping Software version 1.8 (SoftGenetics, State College, Pennsylvania, USA).

**Population genetics parameters in three further species from Veronica subsect. Pentasepalae**—The first comprehensive phylogenetic analysis of Veronica subsect. Pentasepalae based on DNA sequence data revealed four main clades each corresponding to a broad geographic area (Rojas-Andrés et al., 2015). Thus, for the characterization of the microsatellite markers, diploid populations corresponding to species from different clades were selected (Appendix 1): V. orsiniana (core clade), V. javalambrensis (Iberian clade), and V. rosea (North African clade). The Central Asian clade was not considered because no material was available. The mean number of alleles per locus, observed and expected heterozygosities, possible deviations from Hardy–Weinberg equilibrium (HWE; Table 2), and tests for linkage disequilibrium between markers in each population were estimated using Arlequin version 3.5.1.2 (Excoffier and Lischer, 2010).

The number of alleles per locus ranged from one to six, one to 11, and one to nine in the V. orsiniana, V. javalambrensis, and V. rosea populations, respectively. Loci 26, 49, and 52 were monomorphic in V. orsiniana, loci 10 and 52 were monomorphic in V. javalambrensis, and in V. rosea, loci 8 and 13 were monomorphic and locus 49 did not amplify. The observed and expected heterozygosities for all populations are shown in Table 2. Significant deviation from HWE (P < 0.05) was seen for loci 8, 10, 13, and 54 in V. orsiniana, for locus 50 in V. javalambrensis, and for loci 10 and 50 in V. rosea. Linkage disequilibrium showed significance levels below 0.05 after false discovery rate (FDR) correction in two pairwise comparisons (pair 20–52 in V. rosea and pair 27–54 in V. orsiniana).

**Cross-amplification in other species from Veronica subsect. Pentasepalae and 10 subgenera of Veronica**—Cross-amplification performed for these 12 polymorphic loci showed successful results within the expected allele size in two additional species from Veronica subsect. Pentasepalae: V. austrica L. and V. dentata F. W. Schmidt. Tests were also performed for 20 additional species from 10 different subgenera within the large genus Veronica (Table 3). The tests were carried out with the original PCR protocol. The 12 loci tested in agarose gel showed successful amplification of at least several bands. Six of these (8, 10, 13, 19, 26, and 35) showed good amplification results in most samples.

**CONCLUSIONS**

A set of polymorphic microsatellite markers for Veronica subsect. Pentasepalae is reported. Amplification success for these markers in the cross-transferability tests extends their potential usefulness to other subgenera. These markers will be useful for investigating genetic parameters, which may provide essential information for the conservation of threatened species, as well as data on the role of interspecific hybridization in the evolution of the genus.

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Appendix 1. Voucher information for the Veronica samples used in this study.

| Species | Collector no. | Collection country and locality | Geographic coordinates |
|---------|---------------|--------------------------------|------------------------|
| V. austriaca L. (n = 15) | BR94 (SALA) | Croatia, Gračac, Crnopol | 44°15’02.2”N, 15°48’35.5”E |
| V. catarractae G. Forst. (n = 1) | HMM37 (OLD) | cult, Germany ex UK nursery “Botany Plants” stock, Botanical Garden, Oldenburg | NA |
| V. chamaedryoides subsp. chamaedryoides (Bory & Chaub.) M. A. Fisch. (n = 1) | KBch67 (WU) | Greece, Olympia | 37°51’47.0”N, 21°48’45.0”E |
| V. cymbalaria Bodar (n = 1) | DCA403 (WU) | Greece, Vourakis | NA |
| V. cymbalaria (n = 1) | HMM31 (OLD) | Turkey, Alanya Castle | 36°31’58.0”N, 31°59’25.0”E |
| V. cymbalaria (n = 1) | HMM32 (OLD) | Turkey, Selge | 37°13’04.0”N, 31°07’45.0”E |
| V. dentata F. W. Schmidt (n = 14) | BR178 (SALA) | Austria, Niederösterreich, Krems | 48°24’18.1”N, 15°31’04.4”E |
| V. filiformis Sm. (n = 1) | DCA144 (WU) | Germany, Bonn-Venusberg | 50°41’43.0”N, 07°06’10.0”E |
| V. filiformis (n = 1) | DCA954 (MIG) | Turkey, Cam Pass | 41°13’33.0”N, 42°27’44.0”E |
| V. filiformis (n = 1) | DCA892 (MIG) | Turkey, Uzungol | 40°35’00.0”N, 40°19’00.0”E |
| V. fruticans Jacq. (n = 1) | LS1408 (WU) | USA, Seedling. Botanical Garden, New York | NA |
| V. fruticulos L. (n = 1) | DCA71 (BONN) | Germany, Seedling. Botanical Garden, Bonn | NA |
| V. gentianoides Vahl (n = 1) | DCA350 (WU) | Georgia, Terek-Tal | 42°34’51.6”N, 44°25’12.0”E |
| V. gentianoides Sm. (n = 1) | DCA297 (WU) | Georgia, Kreuzpass | 42°31’02.0”N, 44°28’00.0”E |
| V. gentianoides (n = 1) | MO1598 (SALA) | Georgia, Great Caucasus, Monument Bidara | 42°29’33.0”N, 44°27’10.0”E |
| V. hectori Hook. subsp. coarctata (Cheseman) Garn.-Jones (n = 1) | HMM38 (OLD) | cult, Germany ex New Zealand | NA |
| V. incana L. (n = 1) | BF11726 (WU) | Botanical Garden, Bonn | NA |
| V. jacquinii Baumg. (n = 2) | BR108 (SALA) | Bosnia-Herzegovina, Trebinje | 42°41’02.1”N, 18°17’49.2”E |
| V. jacquinii (n = 2) | BR112 (SALA) | Croatia, Dubrovnik, Gromaca | 42°43’28.0”N, 18°01’4.0”E |
| V. jacquinii (n = 1) | SA389 (SALA) | Montenegro, Kotor, Lovćen | 42°25’04.0”N, 18°47’38.8”E |
| V. jacquinii (n = 2) | SA390 (SALA) | Montenegro, Kotor, Lovćen | 42°25’04.0”N, 18°47’38.8”E |
| V. jacquinii (n = 1) | SA391 (SALA) | Montenegro, Zabljak | 43°09’46.0”N, 19°09’03.0”E |
| V. javalambrensis Pau (n = 30) | DP1278 (SALA) | Spain, Burgos. Ciruelos de Cervera | 41°54’50.4”N, 3°29’47.9”W |
| V. missuricensis Raf. subsp. major (Hook.) M. M. Mart. Ort. & Albach (n = 1) | DCA124 (K) | England, Seedling. Botanical Garden, Kew | NA |
| V. ochracea (Ashwiin) Garn.-Jones (n = 1) | HMM39 (OLD) | cult, Germany ex New Zealand | NA |
| V. officinalis L. (n = 1) | DCA114 (K) | Botanical Garden, Bonn | NA |
| V. orbiculata A. Kern. (n = 1) | BR110 (SALA) | Croatia, Pelješac peninsula | 42°56’14.2”N, 17°22’39.5”E |
| V. orbiculata (n = 2) | MO5547 (SALA) | Croatia, Prapatnice | 43°13’16.1”N, 17°21’35.0”E |
| V. orbiculata (n = 1) | SA392 (SALA) | Montenegro, Zabljak | 43°09’49.9”N, 19°09’03.0”E |
| V. orchidea Crantz (n = 1) | KBps57 (WU) | Bulgaria, Lovech | 43°01’59.0”N, 24°18’09.0”E |
| V. orchidea (n = 1) | KBps54 (WU) | Bulgaria, Lovech | 43°10’49.0”N, 24°44’56.0”E |
| V. orchidea (n = 1) | KB847 (WU) | Hungary. Szabolcs-Szatmár-Bereg | 47°45’02.0”N, 21°52’02.0”E |
| V. orsiniana Ten. (n = 30) | MO6056 (SALA) | Spain. Teruel. Iglesuela del Cid | 40°27’35.0”N, 0°18’46.5”W |
| V. panormitana Tineo ex Guss. (n = 1) | HMM29 (OLD) | Turkey, North of Paravallar | 36°40’02.0”N, 31°53’03.0”E |
| V. planetofoiolata G. Simpson & J. S. Thomson (n = 1) | HMM40 (OLD) | New Zealand. Shotover Saddle | 44°31’21.6”S, 168°40’24.0”E |
| V. rosea Desf. (n = 30) | DP1368 (SALA) | Morocco. Meknès-Tafilalet, Midelt | 32°36’21.1”N, 4°48’39.7”W |
| V. salicaroidoides Hook. f. (n = 1) | HMM69 (OLD) | cult, Kew ex New Zealand. Botanical Garden, Kew | NA |
| V. speciosa R. Cunn. ex A. Cunn. (n = 1) | PG2878 (OLD) | cult. New Zealand ex cult. New Zealand. Wellington | NA |
| V. trichadenia Jord. & Fourr. (n = 1) | HMM30 (OLD) | Spain. Mallorca, Camí des Raiguer | NA |
| V. tripillosa L. (n = 1) | DCA434 (OLD) | Germany, Seedling. Botanical Garden, Oldenburg | NA |
| V. vindobonensis M. A. Fisch. (n = 1) | KBch54 (WU) | Hungary. Heves megye | 47°50’19.0”N, 19°57’44.0”E |

Note: n = number of individuals used in the population genetic analyses; NA = not available.

Abbreviations (collector numbers): BF = Bozo Frajman; BR = Blanca M. Rojas-Andrés; DCA = Dirk C. Albach; DP = Daniel Pinto-Carrasco; HMM = Heidi M. Meudt; KB = Katharina E. Bardy; LS = Lena Struve; MO = M. Montserrat Martinez-Ortega; PGJ = Phil Garnock-Jones; SA = Santiago Andrés-Sánchez.

Herbarium specimens are deposited at the herbaria of Universidad de Salamanca (SAL), Universität Wien (WU), University of Bonn (BONN), Royal Botanic Gardens, Kew (K), Johannes Gutenberg-Universität (MIG), and Carl von Ossietzky Universität Oldenburg (OLD); DNA samples are deposited at Biobanco de ADN Vegetal (Universidad de Salamanca) and Carl von Ossietzky Universität Oldenburg (Germany).

Populations used to generate the data included in Appendix 2.
APPENDIX 2. Primers rejected during the study and reason for discarding.

| Locus | F: | R: | Repeat motif | PCR product size | GenBank accession no. | T_a (°C) | Discarding reason |
|-------|----|----|--------------|------------------|-----------------------|----------|------------------|
| 1     | TGAATAGGTTTTGCGTCGAG | (TTG)_6 | 146         | KT005181 | 52 | Suboptimal quality of the sequences |
| 2     | TGGCGACCAAACAAACAAACA | (AT)_5 | 149 | — | — | Unsuccessful amplification |
| 3     | AACAAATCAAGCATACAGCCA | (TA)_5 | 208 | KT005182 | 58 | Suboptimal quality of the sequences |
| 4     | CGCTATGTCATCATTTATGCCGGA | (TC)_14 | 157 | — | — | Unsuccessful amplification |
| 5     | GTCTGAGAAGAAAAACCCCAA | (ACA)_5 | 104 | KT005183 | 50 | Suboptimal quality of the sequences |
| 6     | CGCAATGAGATACAAACACCAA | (AAC)_5 | 92 | KT005184 | 52 | Suboptimal quality of the sequences |
| 7     | GAATCATGATTTGAGGATCCTT | (ATGG)_6 | 140 | — | — | Unsuccessful amplification |
| 8     | GCCAGTAGCCGCTGGTTTTA | (ACA)_5 | 267 | KT005185 | 52 | Unsuccessful amplification in the Iberian clade |
| 9     | TGGTTGTTTGGTTTGTTGGG | (CTT)_6 | 91 | — | — | Unsuccessful amplification |
| 10    | F: | R: | (AAC)_5 | KT005184 | 52 | Suboptimal quality of the sequences |
| 11    | F: | R: | (ATGG)_6 | KT005185 | 52 | Unsuccessful amplification in the Iberian clade |
| 12    | F: | R: | (GTT)_5 | KT005186 | 55 | Unsuccessful amplification in the Iberian clade |
| 13    | AGACTCTACATCCACATCCCA | (GT)_5 | 144 | KT005187 | 52 | Monomorphic |
| 14    | F: | R: | (TG)_5 | KT005188 | 56 | Monomorphic |
| 15    | F: | R: | (TGG)_5 | KT005189 | 54 | Suboptimal quality of the sequences |
| 16    | F: | R: | (GAA)_5 | KT005190 | 52 | Presence of indels |
| 17    | F: | R: | (GA)_5 | KT005191 | 54 | Presence of indels |
| 18    | F: | R: | (GA)_5 | KT005192 | 54 | Suboptimal quality of the sequences |
| 19    | F: | R: | (GA)_5 | KT005193 | 54 | Suboptimal quality of the sequences |
| 20    | F: | R: | (AC)_7 | KT005194 | 52 | Suboptimal quality of the sequences |
| 21    | F: | R: | (AC)_7 | KT005195 | 52 | Suboptimal quality of the sequences |
| 22    | F: | R: | (AC)_7 | KT005196 | 52 | Suboptimal quality of the sequences |
| 23    | F: | R: | (AC)_7 | KT005197 | 52 | Suboptimal quality of the sequences |
| 24    | F: | R: | (AC)_7 | KT005198 | 52 | Suboptimal quality of the sequences |
| 25    | F: | R: | (AC)_7 | KT005199 | 52 | Suboptimal quality of the sequences |
| 26    | F: | R: | (AC)_7 | KT005200 | 52 | Suboptimal quality of the sequences |

http://www.bioone.org/loi/apps
## APPENDIX 2. Continued.

| Locus | Primer sequences (5′−3′) | Repeat motif | PCR product size | GenBank accession no. | $T_a$ (°C) | Discarding reason |
|-------|--------------------------|--------------|------------------|-----------------------|------------|-------------------|
| 43    | F: ACGATAACTTTCCGGTGAA   | (GA)$_{8}$   | 179              | —                     | —          | Unsuccessful amplification |
|       | R: CAAACATTTTTATCATACACAG|              |                  |                       |            |                   |
| 44    | F: CTTTTAAATGCTTTTCTGAGG| (TTG)$_{5}$  | 179              | KT005200              | 52         | Monomorphic       |
|       | R: ATGTCCTTCATAGTAAACGTC|              |                  |                       |            |                   |
| 45    | F: CTATATCTGAATTTTACCTCC | (ACA)$_{6}$  | 174              | KT005201              | 52         | Presence of indels |
|       | R: GAATTATTTAGGTAGACGGA  |              |                  |                       |            |                   |
| 46    | F: AAGCTTGAGTGAATAAATGTT | (GTT)$_{6}$  | 239              | KT005202              | 55         | Presence of indels |
|       | R: AACHTACTACAGCCAAATCAC|              |                  |                       |            |                   |
| 47    | F: AGTAATCATTCCTCAGTCCTCT | (TC)$_{6}$  | 236              | KT005203              | 53         | Monomorphic       |
|       | R: ACACCTTAGTTACATACAAAG |              |                  |                       |            |                   |
| 48    | F: TGACAAATGTACAGCTAGAGG | (TG)$_{8}$   | 246              | KT005204              | 54         | Presence of indels |
|       | R: GATGAGGAGAGGTAGTATG   |              |                  |                       |            |                   |
| 51    | F: ATTGTTGATATGCAGATCTTG | (CA)$_{8}$   | 303              | —                     | —          | Unsuccessful amplification |
|       | R: TTCCATGTAAATTTTACCTACA|              |                  |                       |            |                   |
| 53    | F: GAATACATCTGACAGGAGTCTT | (TC)$_{6}$  | 301              | KT005205              | 52         | Unsuccessful amplification in the Iberian clade |
|       | R: AACGATAGGTCATCAAGAGGA |              |                  |                       |            |                   |

**Note:** — = no information available; $T_a$ = annealing temperature.