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Source: Applications in Plant Sciences, 3(10)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1500059

Applications in Plant Sciences 2015 3(10): 1500059

PRIMER NOTE

CHARACTERIZATION OF 12 POLYMORPHIC SSR MARKERS IN *VERONICA* SUBSECT. *PENTASEPALAE* (PLANTAGINACEAE) AND CROSS-AMPLIFICATION IN 10 OTHER SUBGENERA[1]

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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. orbiculata* 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. orsiniana*, *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* Benth. is a monophyletic diploid-polyploid complex and one of the four subsections currently recognized within the also monophyletic *Veronica* subgen. *Pentasepalae* M. M. Mart. Ort., 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.

[1]Manuscript received 18 May 2015; revision accepted 19 June 2015.

This research was financially supported by the Spanish Ministry of Science and Innovation through the projects CGL2012-32574 and CGL2009-07555. A predoctoral grant to N.L.G. from the Ministry of Education, Culture, and Sport (AP2010-2968) is also acknowledged. We are also deeply grateful to Blanca Rojas-Andrés and Dirk Albach for their continuous support.

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doi:10.3732/apps.1500059

METHODS AND RESULTS

***Microsatellite development*—**For the microsatellite library, silica gel–dried leaves of 12 diploid individuals of *V. jacquinii* Baumg. and *V. orbiculata* A. Kern. were selected from eight different populations (Appendix 1). Ploidy level 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 cetyltrimethylammonium 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*.[a]

Locus	Primer sequences (5′–3′)	Fluorescent dye	Repeat motif	Allele size range (bp)[b]	T_a (°C)	GenBank accession no.
8	F: TGATGTGACTGATTGGGTCAG	5-FAM	$(TGA)_5$	92–95	55	KR698358
	R: TTACCTCCTCATCACTCCCC					
10	F: TGAACAACACACAGGTTCAATTC	5-FAM	$(AG)_9$	113–119	55	KR698359
	R: GGCTAGAAGTTGTGAAGAAGGG					
13	F: GCTTTTCTCGGTGAAAGGGT	PET	$(TGAT)_5$	113–133	58	KR698360
	R: CACCATAATCCACAGCCTGA					
19	F: TCGAAACTTATTCGGCAACG	5-FAM	$(ATT)_5$	133–157	55	KR698361
	R: GACTCACGAGTTTGGAAGCG					
20	F: TGGAGACCAAAATTCAACCC	PET	$(AC)_{11}$	93–135	52	KR698362
	R: TCTTGTCTCCTACTCTCCTCCG					
26	F: ATGTCGACGTGTCAACTCCA	NED	$(CAA)_6$	87–102	56	KR698363
	R: CACTTGTTTCCACAGCTGGC					
27	F: TATGGGAGACGACATGGTCA	PET	$(TTGTG)_6$	201–221	55	KR698364
	R: CTCCCTTTCGTAGCAACACC					
35	F: CATTTAATGGTATCCGATGCG	NED	$(TATC)_7$	106–130	52	KR698365
	R: TCGCTTTTCGATTTCTTCGT					
49	F: GGATGCTTTATTTTGTCTTGT	VIC	$(TGGA)_5$	222–242	52	KR698366
	R: TGTTACGACATTTATGGTGATT					
50	F: TGTGATGCACAGAGTTTTAGTT	VIC	$(AGA)_6$	400–460	50	KR698367
	R: TGAAAACATAACACCTCGATAA					
52	F: ATAAAAACATCCATACTTTCCG	VIC	$(GTT)_5$	358–391	52	KR698368
	R: GTTAACCGCCAGTCTAACTAAT					
54	F: CCAAATATCAAATGATACCACA	NED	$(AC)_{13}$	283–301	52	KR698369
	R: TCGTAAAATTACGTCATCAAGA					

Note: T_a = annealing temperature.
[a]All values are based on 90 samples from three *Veronica* populations.
[b]Range of fragment sizes does not include the M13 tail (5′-TGTAAAACGACGGCCAGT-3′) attached to the forward primer.

AAC, AAG, AGG, ACG, 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 195 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 *V. jacquinii–V. orbiculata*. PCRs were performed in a total volume of 15 µL, which 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 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).

In a second step, those primers that were polymorphic in the *V. 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′-TGTAAAACGACGGCCAGT-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 M13 primer, 0.04 mM of forward primer, 0.75 units GoTaq DNA Polymerase,

TABLE 2. Results of initial primer screening of polymorphic loci in three populations corresponding to three different taxa belonging to *Veronica* subsect. *Pentasepalae*.[a]

	V. orsiniana (n = 30)				*V. javalambrensis* (n = 30)				*V. rosea* (n = 30)			
Locus	A	H_o	H_e	HWE[b]	A	H_o	H_e	HWE[b]	A	H_o	H_e	HWE[b]
8	2	0.933	0.506	0.000***	2	0.167	0.155	1.000 ns	1			
10	2	0.000	0.066	0.017*	1				3	0.033	0.097	0.017*
13	2	0.167	0.440	0.001***	6	0.500	0.500	0.388 ns	1			
19	2	0.333	0.488	0.125 ns	4	0.700	0.697	0.852 ns	4	0.233	0.298	0.968 ns
20	4	0.700	0.525	0.140 ns	10	0.767	0.818	0.077 ns	9	0.690	0.736	0.144 ns
26	1				3	0.433	0.432	1.000 ns	5	0.690	0.743	0.391 ns
27	3	0.500	0.560	0.290 ns	3	0.483	0.381	0.448 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
49	1				6	0.633	0.742	0.061 ns	—	—	—	—
50	3	0.233	0.216	1.000 ns	11	0.567	0.785	0.017*	4	0.037	0.240	0.000***
52	1				1				3	0.136	0.210	0.222 ns
54	6	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_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium probabilities; n = number of individuals sampled.
[a]See Appendix 1 for locality and voucher information for each population.
[b]Deviations from HWE were not statistically significant (ns) and statistically significant at $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P \leq 0.001$.

TABLE 3. Amplification success of all microsatellite primers across 20 species from 10 subgenera of *Veronica*.

Subgenera	Collector no.[a,b]	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	—	s	—	+	—	—	—	—
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.) M. M. Mart. Ort. & Albach	DCA403	*V. cymbalaria*	+	+	+	s	w	s	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	—	+	—	—	—	—
Veronica subg. *Cochlidiosperma*	HMM30	*V. trichadena*	+	s	+	s	—	+	—	w	—	—	—	—
Veronica subg. *Pellidosperma* (E. B. J. Lehm.) M. M. Mart. Ort., Albach & M. A. Fisch.	DCAs434	*V. triphyllos*	+	+	+	w	s	s	w	s	—	—	+	w
Veronica subg. *Pocilla* (Dumort.) M. M. Mart. Ort., Albach & M. A. Fisch.	DCA144	*V. filiformis*	w	+	+	s	w	s	w	+	—	—	—	—
Veronica subg. *Pocilla*	DCA954	*V. filiformis*	s	+	+	s	w	s	+	+	—	—	v	s
Veronica subg. *Pocilla*	DCA892	*V. filiformis*	s	+	+	s	w	+	+	+	—	—	—	s
Veronica subg. *Pseudolysimachium* (W. D. J. Koch) M. M. Mart. Ort., Albach & M. A. Fisch.	KB847	*V. orchidea*	s	+	w	s	+	s	+	+	—	—	—	s
Veronica subg. *Pseudolysimachium*	KBps54	*V. orchidea*	+	s	+	+	—	+	—	+	—	—	—	w
Veronica subg. *Pseudolysimachium*	KBps57	*V. orchidea*	w	s	+	w	—	+	—	+	—	—	—	w
Veronica subg. *Pseudolysimachium*	BF11726	*V. incana*	w	s	+	w	—	+	—	+	—	—	—	—
Veronica subg. *Pseudoveronica* J. B. Armstr.	PGJ2878	*V. speciosa*	s	s	+	s	+	s	s	s	—	—	—	—
Veronica subg. *Pseudoveronica*	HMM69	*V. salicornioides*	s	s	+	s	+	s	s	s	—	—	—	—
Veronica subg. *Pseudoveronica*	HMM38	*V. hectori* subsp. *coarctata*	w	s	+	s	+	s	s	w	—	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
Veronica subg. *Pseudoveronica*	HMM37	*V. catarractae*	s	s	w	s	+	s	s	+	—	s	—	s
Veronica subg. *Stenocarpon* (Boriss.) M. M. Mart. Ort., Albach & M. A. Fisch.	LS1408	*V. fruticans*	s	s	s	+	s	s	+	s	—	w	+	+
Veronica subg. *Stenocarpon*	DCA71	*V. fruticulosa*	s	+	+	+	s	s	+	s	—	+	+	+
Veronica subg. *Synthyris* (Benth.) M. M. Mart. Ort., Albach & M. A. Fisch.	DCA124	*V. missurica*	w	+	w	+	+	+	+	s	—	—	+	w
Veronica subg. *Veronica*	DCA114	*V. officinalis*	w	w	s	w	w	+	w	w	—	—	+	w

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

[a]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.

[b]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. austriaca* 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. (Herbarium code)[a,b]	Collection country and locality	Geographic coordinates
V. austriaca L. (*n* = 15)	BR94 (SALA)	Croatia. Gračac, Crnopac	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. chamaedrys L. 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 Bodard (*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 (MJG)	Turkey. Cam Pass	41°13′33.0″N, 42°27′44.0″E
V. filiformis (*n* = 1)	DCA892 (MJG)	Turkey. Uzungoel	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. fruticulosa 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 (*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. f. subsp. *coarctata* (Cheeseman) Garn.-Jones (*n* = 1)	HMM38 (OLD)	cult. Germany ex New Zealand. Botanical Garden, Bonn	NA
V. incana L. (*n* = 1)	BF11726 (WU)	Serbia. Grgurevci	45°06′36.0″N, 19°40′05.0″E
V. jacquinii Baumg. (*n* = 2)[c]	BR108 (SALA)	Bosnia-Herzegovina. Trebinje	42°41′02.1″N, 18°17′49.2″E
V. jacquinii (*n* = 2)[c]	BR112 (SALA)	Croatia. Dubrovnik, Gromača	42°43′28.0″N, 18°01′4.0″E
V. jacquinii (*n* = 1)[c]	SA389 (SALA)	Montenegro. Kotor, Lovćen	42°25′04.9″N, 18°47′38.8″E
V. jacquinii (*n* = 2)[c]	SA390 (SALA)	Montenegro. Kotor, Lovćen	42°25′04.9″N, 18°47′38.8″E
V. jacquinii (*n* = 1)[c]	SA391 (SALA)	Montenegro. Žabljak	43°09′49.6″N, 19°09′00.3″E
V. javalambrensis Pau (*n* = 30)[c]	DP1278 (SALA)	Spain. Burgos. Ciruelos de Cervera	41°54′50.4″N, 3°29′47.9″W
V. missurica Raf. subsp. *major* (Hook.) M. M. Mart. Ort. & Albach (*n* = 1)	DCA124 (K)	England. Seedling. Botanical Garden, Kew	NA
V. ochracea (Ashwin) Garn.-Jones (*n* = 1)	HMM39 (OLD)	cult. Germany ex New Zealand. Botanical Garden, Bonn	NA
V. officinalis L. (*n* = 1)	DCA114 (K)	England. Seedling. Botanical Garden, Kew	NA
V. orbiculata A. Kern. (*n* = 1)[a]	BR110 (SALA)	Croatia. Pelješac peninsula	42°56′14.2″N, 17°22′39.5″E
V. orbiculata (*n* = 2)[c]	MO5547 (SALA)	Croatia. Prapatnice	43°13′16.1″N, 17°21′35.0″E
V. orbiculata (*n* = 1)[c]	SA392 (SALA)	Montenegro. Žabljak	43°09′49.6″N, 19°09′00.3″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)[c]	MO6056 (SALA)	Spain. Teruel. Iglesuela del Cid	40°27′35.9″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. planopetiolata 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)[c]	DP1368 (SALA)	Morocco. Meknès-Tafilalet, Midelt	32°36′21.1″N, 4°48′39.7″W
V. salicornioides Hook. f. (*n* = 1)	HMM69 (OLD)	cult. Kew ex New Zealand. Botanical Garden, Kew	NA
V. speciosa R. Cunn. ex A. Cunn. (*n* = 1)	PGJ2878 (OLD)	cult. New Zealand ex cult. New Zealand. Wellington	NA
V. trichadena Jord. & Fourr. (*n* = 1)	HMM30 (OLD)	Spain. Mallorca, Camí des Raiguer	NA
V. triphyllos L. (*n* = 1)	DCAs434 (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.

[a] 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 Struwe; MO = M. Montserrat Martínez-Ortega; PGJ = Phil Garnock-Jones; SA = Santiago Andrés-Sánchez.

[b] Herbarium specimens are deposited at the herbaria of Universidad de Salamanca (SALA), Universität Wien (WU), University of Bonn (BONN), Royal Botanic Gardens, Kew (K), Johannes Gutenberg-Universität (MJG), 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).

[c] Populations used to generate the data included in Appendix 2.

Appendix 2. Primers rejected during the study and reason for discarding.

Locus	Primer sequences (5′–3′)	Repeat motif	PCR product size	GenBank accession no.	T_a (°C)	Discarding reason
1	F: TGATAGGGTTTGTGCGTGAG R: TGTCGACCAAACCAAAACAA	$(TTG)_6$	146	KT005181	52	Suboptimal quality of the sequences
2	F: CCCTTTGGAGTTGTTATGATCG R: GAATGAACGGTTTAAGTGGACA	$(AT)_5$	149	—	—	Unsuccessful amplification
3	F: AACAAATCATAAGCAATGCCA R: CGCTAGTGTCATCATGTTATGC	$(TA)_5$	208	KT005182	58	Monomorphic
4	F: AATTAAATTTCGCGGATCCTT R: CGGTCTTACCAATGGCAGAT	$(TC)_{14}$	157	—	—	Unsuccessful amplification
5	F: GCTGGAAAGAAAACCCAACA R: TTGCATTGGATTTTGAACCA	$(ACA)_5$	104	KT005183	50	Suboptimal quality of the sequences
6	F: CGAAATCAGAATCAACACCAA R: GAATCATCGATTGGGATCTTT	$(AAC)_6$	92	KT005184	52	Suboptimal quality of the sequences
7	F: CCCGAGTAGCGCTTGTTTTA R: CACGAGTATGGGACGATTCA	$(TC)_8$	152	—	—	Unsuccessful amplification
9	F: GCACGGAAACAACATGAACA R: TCCCCATCATAATCACAATCA	$(AG)_8$	267	KT005185	52	Unsuccessful amplification in the Iberian clade
11	F: TTGTTGGTTTTGGTTTGTGG R: GATGAACTCCAATCTACCCCA	$(CTT)_{12}$	91	—	—	Unsuccessful amplification
12	F: GCCACGGAGACTCAGGTTAG R: TGACGAATAGCAATAGACAACGA	$(GTT)_5$	132	KT005186	55	Suboptimal quality of the sequences
14	F: AAAGATAATTGTCCTAAAGTTAAGGGG R: GCAGCATTATGCAGGTAGATT	$(ATGG)_6$	140	—	—	Unsuccessful amplification
15	F: ACGCTTGAACGCGTCTAACA R: AGATCCCCACTCACGATCTC	$(GT)_6$	144	KT005187	54	Monomorphic
16	F: ATCGAGGACGGATTTAGGCT R: AAGTGCCCTTTCCTCCAAAC	$(GTA)_5$	113	KT005188	56	Monomorphic
17	F: GAGTGATCGAAAGATTGCATTAAG R: TCCTCCCTAATTCCTCCGAC	$(GTG)_6$	148	KT005189	54	Suboptimal quality of the sequences
18	F: TTGAATATCAGGATCTTGTGCG R: AAGTAATATGTCCATAAGTTCATCAGG	$(TCT)_6$	91	KT005190	58	Suboptimal quality of the sequences
21	F: AGAGGATGAAGACTCAGGCG R: TGTCAGCTTTGGTGGAAGAA	$(GAA)_9$	140	—	—	Unsuccessful amplification
22	F: GACGACGATCATCCAGATCC R: CCGATTTCCTTTCGAATCAT	$(AGA)_6$	147	KT005191	52	Presence of indels
23	F: AAACTTGTGAAACTGTTTGAATGG R: ATGCTCAGCGGAAGTATTTGA	$(CA)_5$	90	—	—	Unsuccessful amplification
24	F: TTCCGATATTTCCGTTCTGC R: CCATTCTACCCTCCGAACAA	$(GAG)_6$	142	KT005192	52	Presence of indels
25	F: GCACAAGGTAGCATTTGCATT R: AGGGCGGGTAAAGGATAGAA	$(TTG)_9$	142	—	—	Unsuccessful amplification
28	F: GTGTTCGTGTTTTAAATTTGCTT R: TCACTCATATACCTAGTGACTGAACTG	$(GAG)_{11}$	141	—	—	Unsuccessful amplification
29	F: TTGAATCCATTTCTTATTGGTTTG R: CAATCGTGGTAACACATCATGG	$(TTC)_7$	90	KT005193	53	Unsuccessful amplification in the Iberian clade
30	F: CTTCCTTACCTCACCTCACTCTG R: TGGTGTTTTGTTGATAGATTGATT	$(CAT)_5$	91	KT005194	53	Suboptimal quality of the sequences
31	F: GCCATTGCCTTGTTTTGAGT R: CATCAACCATGATCCATCCA	$(GA)_9$	91	—	—	Unsuccessful amplification
32	F: ATTGAGCGACACTCGTCAGA R: CAATGGCTTTAAATGAATCCC	$(AC)_7$	140	KT005195	52	Monomorphic
33	F: TTCAGCTCATGACCAAGAACA R: CAAATAGGGCATTCCGACAT	$(AAG)_6$	123	KT005196	50	Unsuccessful amplification in the Iberian clade
34	F: TAAACAAACAGATTGGTGGTCG R: CCTTATGTCACTGAAAACCTACCT	$(TAA)_6$	190	KT005197	54	Unsuccessful amplification in the Iberian clade
36	F: CGGTGCCAAATTAAGATATTG R: GCGGTGAAGAAAGGTTTTGA	$(ACTC)_5$	182	—	—	Unsuccessful amplification
37	F: TGCACCCCTACTCGAGAAAT R: TCCATTTAATTGTAAGCCCCA	$(CT)_8$	120	—	—	Unsuccessful amplification
38	F: ACAGGTTGTGCGGAAGAAGT R: GTGTGCCAACAAATCAAGGA	$(TGT)_9$	155	KT005198	52	Suboptimal quality of the sequences
39	F: GAAAAGAATTACCAACACGC R: TTAAGGCCTAGCTAGCAGAA	$(AAAG)_6$	93	—	—	Unsuccessful amplification
40	F: ATCTCCAAAACTCAGATCCA R: TTAAGGCCTAGCTAGCAGAA	$(AAC)_6$	86	—	—	Unsuccessful amplification
41	F: TCATAGCTTCTTCTCTTCGG R: TATGATGGCCTTCAAAACAT	$(CTT)_5$	85	—	—	Unsuccessful amplification
42	F: TGTATTATTCTATGAGACGCCA R: GTGAGAAGACATATGAAAAGCA	$(TG)_{16}$	193	KT005199	52	Suboptimal quality of the sequences

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: CAACCATTTTCTTCATACACAG					
44	F: CTTTTAAATGTCTTTCTGGAGG	$(TTG)_5$	179	KT005200	52	Monomorphic
	R: ATGTCCTTCATAGTAAACGTCC					
45	F: CTTATCCTTGAATTTCATCTCC	$(ACA)_6$	174	KT005201	52	Presence of indels
	R: GATTATTTTACGGTTAGACGGA					
46	F: AAGCTTGAGTGGATTAAATGTT	$(GTT)_6$	239	KT005202	55	Presence of indels
	R: AACTCTTACCACCTCAAATCAC					
47	F: AGTAATCAATTCTCACTTGGCT	$(TC)_5$	236	KT005203	53	Monomorphic
	R: ACAACCCTAGTTCATACCAAAG					
48	F: TGAACAAATGTACAGCTAGAGG	$(TG)_9$	246	KT005204	54	Presence of indels
	R: GATGAGGAGAAGGAGTGTATGT					
51	F: ATTGTTGTATATGCGAATCTTG	$(CA)_8$	303	—	—	Unsuccessful amplification
	R: TTCCATGTAAATTTCACTACCA					
53	F: GAATACATTCAGACCACGTCTT	$(TC)_8$	301	KT005205	52	Unsuccessful amplification in the Iberian clade
	R: AAACGATAGAGTCTCAAGAGGA					

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