

DEVELOPMENT OF 14 MICROSATELLITE MARKERS IN *ODONTITES VERNUS* S.L. (OROBANCHACEAE) AND CROSS-AMPLIFICATION IN RELATED TAXA¹

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- *Premise of the study:* Microsatellite primers were developed for the first time in the root hemiparasite herb *Odontites vernus* (Orobanchaceae). These markers will be useful to investigate the role of polyploidization in the evolution of this diploid-tetraploid complex, as well as the extent of gene flow between different ploidy levels.
- *Methods and Results:* Fourteen polymorphic and reproducible loci were identified and optimized from *O. vernus* using a microsatellite-enriched library and 454 Junior sequencing. The set of primers amplified di- to pentanucleotide repeats and showed two to 13 alleles per locus. Transferability was tested in 30 taxa (19 belonging to *Odontites* and 11 from eight other genera of Orobanchaceae tribe Rhinanthae).
- *Conclusions:* The results indicate the utility of the newly developed microsatellites in *O. vernus* and several other species, which will be useful for taxon delimitation and conservation genetics studies.

Key words: conservation; diploid-tetraploid complex; microsatellite; *Odontites vernus*; Orobanchaceae; Rhinanthae.

The predominantly Mediterranean genus *Odontites* Ludw. (Orobanchaceae; Bennett and Mathews, 2006) comprises ca. 26 species of annual and perennial root hemiparasites (Bolliger, 1996) growing in grasslands, shrublands, and wood edges. It includes weeds (Parker, 2013), as well as species listed on national and regional catalogs of endangered plants (e.g., López Udías and Fábregat Llucca, 2010), registered on the International Union for the Conservation of Nature Red List (<http://www.iucnredlist.org/>), or with narrow distribution areas (Bolliger, 1996).

The *O. vernus* (Bellardi) Dumort. group, which includes three species, is the most widespread of the genus, occupying the temperate regions of Eurasia with one population in northern Morocco (Bolliger, 1996). However, phylogenetic relationships and evolutionary patterns within the group remain largely unclear due to a complex interplay between the diploid-tetraploid cytotypic variation and seasonal ecotypes differing in morphology (Koutecký et al., 2012). *Odontites vernus* sensu lato (s.l.;

Rico, 2009) includes diploid and tetraploid individuals. The latter are probably of autopolyploid origin, as no distinct subgenomes were found in the karyotype (Delgado et al., 2015) and morphology is not intermediate between any two known diploid species. However, the hypothesis of an autopolyploid origin has not been addressed using genetic markers. Furthermore, it is not clear whether some levels of gene flow are maintained in locations where diploids and tetraploids co-occur (Snogerup, 1983; Koutecký et al., 2012). Although it is known that *O. vernus* can self-pollinate (Nilsson and Alves-dos-Santos, 2009), inbreeding rates in populations remain unknown. Therefore, genetic markers are needed to study gene flow patterns and how populations of *O. vernus* are connected. Furthermore, the transferability of the loci to other species of the genus would bring new information for taxonomic revision of *Odontites* species and conservation of endemic and/or threatened taxa.

METHODS AND RESULTS

Microsatellite development—Silica gel-dried leaves of two diploid individuals of *O. vernus* (see Appendix 1 for voucher information) were selected for genomic DNA extraction using Invisorb Spin Plant Mini Kit (Invitex, Berlin, Germany). Ploidy level was checked with a CyFlow flow cytometer (Partec GmbH, Münster, Germany), using ‘Woody Plant Buffer’ (WPB; Loureiro et al., 2007) and *Solanum pseudocapsicum* L. as the internal standard (Temsch et al., 2010). DNA extraction was enriched with AC, AG, TGT, and CCT motifs following Nunome et al. (2006). The resulting microsatellite library was sequenced using a 454 GS Junior Sequencer (454 Life Sciences, a Roche Company, Branford, Connecticut, USA). Analyses with QDD software (Megléczy et al., 2010) revealed 4335 sequence reads with microsatellite motifs (from a total of 16,050), and primer pairs were designed for 169 regions. A set of 36 primer pairs with low penalty, different lengths, and containing different repeat

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TABLE 1. Characteristics of 14 polymorphic microsatellite loci developed in *Odontites vernus*.

Locus	Primer sequences (5'-3')	Fluorescent label	Repeat motif	Allele size range (bp) ^a	A	Indel detected	T _a (°C)	T _d (°C)	GenBank accession no. ^b
Ov-2	F: CCCAAGTTTGTAAATTGGATCG R: GAACTGCAGCTGGAACTCTTA	VIC	(AATT) ₉	171–213	11	Y	54	94	KI777566–KI777574
Ov-5	F: ATTAGTACAAACACACACGAGGG R: ATACTCGGCACTTGGCAATTC	VIC	(TA) ₄ -(CA) ₈	178–190	6	N	55	94	KI777577–KI777579
Ov-6	F: CACTCTCCCACTTCTTGATT R: TCAGAAATGGGGTATGAGAAA	NED	(AGC) ₆	92–116	13	Y	54	94	KI777580–KI777583
Ov-10	F: TGAATAATGTTTCACTCCATAC R: CACACTTTAGCTATGTGCC	PET	(AGT) ₆	213–217	4	Y	55	94	KI777587–KI777590
Ov-15	F: CTAGGTTTGGGAATGTGGT R: CCTAGTACCAGATACCATCC	NED	(ACT) ₅	93–108	11	Y	54	*	KI777593–KI777596
Ov-17	F: TATCGATCCACTCGTGAACAC R: TTCAGATCACGGTACAGATTCC	PET	(AAAAAC) ₅	309–327	4	Y	55	94	KI777597–KI777599
Ov-19	F: GAGGAGATGAGGATTCGATA R: CCCACATTTCAITTTACTCTCC	5-FAM	(AGGG) ₅	85–94	7	—	55	94	KI777600
Ov-20	F: GAGGAGACCCAAATAACAAAATTA R: AATTTAAGCACCATGTTGAA	PET	(AGTT) ₃	98–122	5	Y	55	83	KI777601–KI777603
Ov-21	F: GATCCATTAGCAATGGACTTT R: TCCAGGTCAAACAGTGAACAAC	NED	(AG) ₁₁	238–274	13	N	55	94	KI777604–KI777613
Ov-25	F: CTGCCATAGATAACATGCCAAA R: CCCATGCCGAGAAAAG	5-FAM	(TA) ₃ -(CA) ₉	185–196	8	Y	55	94	KI777617–KI777620
Ov-28	F: ACAAGATTTCCCTCCCTGTC R: ATCCATGTGAGCAATGATGAAA	5-FAM	(AC) ₆	227–258	8	Y	55	*	KI777622–KI777626
Ov-30	F: TTCGGATATCGAATAAAAATGGG R: TCGCAATGTTTCCCTGTTGTTAC	NED	(AC) ₅	249–257	6	Y	55	94	KI777627–KI777629
Ov-33	F: CCTTGAAACATACAGAAACTACAACAA R: TGATTACTATGCAACCACTGCC	VIC	(AC) ₅	367–387	3	Y	55	94	KI777631–KI777633
Ov-35	F: TCAAATTCATTAGAATCGGTCA R: CTATTTGACCAATGAGCTCCACC	PET	(ACC) ₅	310–313	2	N	55	83	KI777634–KI777635

Note: A = number of alleles; T_a = optimal annealing temperature; T_d = optimal denaturation temperature.

^aRange of fragment sizes included the M13(–21) tail attached to the forward primer. Sizes were taken from GeneMarker allele calls.

^bMore than one accession per locus (except for Ov-19) in order to check imperfect microsatellite motifs and/or indel presence.

*No differences in genotyping between both T_d tested.

TABLE 2. Results of initial screening of within-population variation in two populations of *Odontites vernus*.

Locus	Tejada (n = 30)				SMA diploids (n = 32)				SMA tetraploids (n = 36)		
	A	H _o	H _e	HWE ^a	A	H _o	H _e	HWE ^a	A	A _{per ind.}	H _o ^b
Ov-2	2	0.16667	0.34520	0.01190*	3	0.06250	0.63641	0.00000***	1	—	—
Ov-5	1	—	—	—	1	—	—	—	2	2	1.00
Ov-6	1	—	—	—	1	—	—	—	3	3	1.00
Ov-10	1	—	—	—	2	0.15625	0.48363	0.00013***	1	—	—
Ov-15	4	0.10000	0.29774	0.00000***	2	0.18750	0.49008	0.00080***	3	3	1.00
Ov-19	2	0.00000	0.12655	0.00090***	1	—	—	—	2	2	1.00
Ov-20	1	—	—	—	2	0.18750	0.50000	0.00068***	1	—	—
Ov-21	2	0.10000	0.46271	0.00003***	5	0.21875	0.65278	0.00000***	1	—	—
Ov-28	1	—	—	—	1	—	—	—	3	3	1.00
Ov-33	1	—	—	—	1	—	—	—	2	2	1.00

Note: — = monomorphic loci; A = number of alleles; A_{per ind.} = maximum number of alleles in a single individual; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium probabilities; n = number of individuals sampled.

^aDeviations from HWE were statistically significant at *P < 0.05 and ***P < 0.001. Note that there were no deviations at P < 0.01.

^bAs it is not possible to calculate H_o accurately for tetraploids, the proportion of individuals with more than one allele is shown.

motifs was tested. Amplification was evaluated in four diploid and three tetraploid individuals of *O. vernus*. PCRs were performed in 12.5-μL reactions, which contained 45.5 ng of DNA, 1× PCR buffer (Biotools, Madrid, Spain), 1.5 mM MgCl₂ (Biotools), 0.2 mM of each dNTP (Life Technologies, Carlsbad, California, USA), 0.33 mM of each primer (Eurofins, Ebersberg, Germany), and 0.5 unit of DNA Polymerase (Biotools), using 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 primer-specific annealing temperature, and 50 s at 72°C; and a final extension of 15 min at 72°C. PCR products were visualized on a 2.5% agarose gel.

PCR products were sequenced by Macrogen Europe (Amsterdam, The Netherlands), and the obtained sequences were checked for homology to the expected region. Consistent amplification and levels of polymorphisms were analyzed in gel images. Eighteen loci were selected (see Appendix 2 for discarding reasons) and tested on 140 *O. vernus* samples using a three-primer PCR protocol (Schuelke, 2000) with the universal primer M13(–21) 5′-TGTA-AAACGACGGCCAGT-3′ marked with 5-FAM, VIC, NED, or PET fluorescent dyes (Life Technologies; Table 1). The PCR mix was as described above, except that 0.2 mM of each reverse and fluorescent-labeled M13 primer and 0.08 mM of the forward primer were used. Cycling conditions were also 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. Pooled PCR products were run on an ABI 3730 Capillary Sequencer (Life Technologies) using GeneScan 500 LIZ Size Standard (Life Technologies). Electropherograms were analyzed with GeneMarker AFLP/Genotyping Software version 1.8 (SoftGenetics, State College, Pennsylvania, USA). Three loci were discarded due to genotyping difficulties, and an additional one was monomorphic. Because lengths of some alleles differed from expected sizes, alleles found in homozygous individuals were sequenced to verify indel presence and/or imperfect microsatellite motifs. Indel presence was confirmed in all but three loci (Ov-19, Ov-21, and Ov-35), and imperfect microsatellite motifs were confirmed in two loci (Ov-5 and Ov-25). Additionally, denaturation temperature (T_d) was reduced to 83°C to test if lower T_d improved genotyping (Olejniczak and Krzyzosiak, 2006). Of the remaining 14 loci, T_d = 83 produced better results for two loci, in two cases there were no differences, and in 10 loci there was reduced scorability, contrary to expectations.

Population genetic parameters in two populations of *Odontites vernus*—

Two populations were selected to obtain population genetic parameters that could be illustrative of performance in two different situations. In one population (Tejada), all sampled individuals were diploids, but in the other one (San Miguel del Arroyo [SMA]) 32 were diploids and 36 were tetraploids. The number of alleles per locus, observed and expected heterozygosity, significance of deviation from Hardy–Weinberg equilibrium (HWE; Table 2), and test for linkage disequilibrium between markers were estimated using Arlequin version 3.5.1.2 (Excoffier and Lischer, 2010). To perform those analyses, allele sizes were not transformed into number of repeats, and exact allele dosage was not estimated in tetraploids. In SMA, these parameters were calculated only for diploids. The number of alleles per locus ranged from two to 13 in the complete data set (Table 1), but varied from one to five in the two selected populations (Table 2). Four loci were monomorphic in both populations, and four

to six were polymorphic in the studied populations. Significant deviation from HWE (P < 0.05) was found in all loci probably due to inbreeding, as recorded in the closely related genus *Euphrasia* L. (French et al., 2003). Linkage disequilibrium was significant after Bonferroni correction in all pairwise comparisons, except those involving allele Ov-19 and the pair Ov-10/Ov-15. Regarding alleles related to ploidy levels, almost all alleles in every locus are shared between ploidy levels overall. But in the SMA samples, there are six loci (Ov-5, Ov-19, Ov-21, Ov-28, Ov-30, Ov-33) that differentiate ploidies unequivocally.

Cross-amplification in other *Odontites* species and related genera—The 18 selected loci were tested in 19 *Odontites* taxa and 11 other taxa from eight related genera using the PCR conditions described above. Fragment separation results (Table 3) were promising in closely related species (*O. corsicus* (Loisel.) G. Don, *O. hollianus* (Lowe) Benth., *O. luteus* (L.) Clairv., *O. kaliformis* (Pourel) Willd.) Pau, and *O. recordonii* Burnat & Barbey) because they amplify in 13 to 17 loci, and sometimes showed more than one allele, despite a small sample size (n = 4). Furthermore, good results were obtained for several other taxa–locus combinations. Development of species-specific PCR protocols could improve these results, especially in some other *Odontites* species (i.e., *O. bolligeri* E. Rico, L. Delgado & Herrero, *O. pyrenaicus* (Bubani) Rothm., and *O. cebennensis* H. J. Coste & Soulié).

CONCLUSIONS

A set of polymorphic microsatellite markers for *O. vernus* is reported for the first time. Successful results for these loci in the cross-amplification tests extend their potential usefulness to other closely related taxa. These markers will be useful for investigating genetic diversity in threatened species, self-pollination rates, origin and evolution of polyploidy, and ecotypic variation and local adaptation in populations.

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TABLE 3. Results of cross-amplification within the genus *Odontites* and related genera.^a

Species ^b	n	Ov-2	Ov-3 ^c	Ov-5	Ov-6	Ov-10	Ov-12 ^c	Ov-15	Ov-17	Ov-19	Ov-20	Ov-21	Ov-25	Ov-26 ^e	Ov-28	Ov-30	Ov-32 ^c	Ov-33	Ov-35
<i>B. inaequalis</i> Benth.	1								0/0/1 (1)	0/0/1 (1)	0/0/1 (1)	0/0/1 (1)				0/0/1 (1)			
<i>Ba. rameauana</i> (Emb.) Bolliger	4	2/1/1 (2)	3/0/1 (1)			1/0/3 (4)			0/0/4 (2)	0/0/4 (2)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	0/0/4 (3)				1/3/0 (2)*	
<i>Be. trixago</i> (L.) All.	2								0/0/2 (1)	0/0/2 (1)	0/0/2 (2)	0/0/2 (1)							
<i>Eu. antarctica</i> Benth.	1								0/0/1 (1)	0/0/1 (1)	0/0/1 (1)	0/0/1 (1)							
<i>Eu. hirtella</i> Jord. ex Reut.	1	0/0/1 (1)							0/0/1 (1)	0/0/1 (1)	0/0/1 (1)	0/0/1 (1)							
<i>Ma. longiflorum</i> (Lam.) Rothm.	4	3/0/1 (1)	3/0/1 (1)	0/0/4 (1)		3/0/1 (2)			0/2/2 (1)	0/2/2 (1)	0/0/4 (2)	0/0/4 (2)	0/0/4 (2)	0/1/3 (1)*	3/0/1 (1)			3/0/1 (1)*	
<i>No. asperrima</i> (Link) Benedi & Herrero	4			0/0/4 (1)		1/0/3 (1)			0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)						2/2/0 (2)
<i>No. spicata</i> (Ramond)	4	3/0/1 (1)	3/1/0 (1)			0/0/4 (1)			0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)						1/2/1 (1)
Bolliger & Molau																			
<i>Od. virgata</i> (Link) Rothm.	4	2/0/2 (2)				3/0/1 (1)			0/0/4 (1)	0/0/4 (1)	0/0/4 (2)	0/0/4 (2)	0/0/4 (2)	3/0/1 (1)					
<i>O. bocconii</i> (Guss.) Walp.	4	2/0/2 (1)	3/0/1 (1)		0/0/4 (3)*				0/0/4 (2)	0/0/4 (2)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	2/2/0 (1)	0/0/4 (3)			1/2/1 (1)*	
<i>O. bolligeri</i> E. Rico, L. Delgado & Herrero	4	3/0/1 (1)	2/0/2 (1)		1/0/3 (1)	0/0/4 (1)			0/0/4 (2)	0/0/4 (2)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	0/2/2 (4)	2/2/0 (1)	3/1/0 (1)	0/0/4 (1)*		
<i>O. cebnensis</i> H. J. Coste & Soulié	4	3/0/1 (1)		3/1/0 (1)	0/0/4 (1)	0/0/4 (3)			1/1/2 (1)	0/0/4 (2)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	0/0/4 (2)	0/3/1 (3)		0/0/4 (1)	
<i>O. corsicus</i> (Loisel.) G. Don	4	0/0/4 (1)	0/0/4 (3)	0/0/4 (1)	0/0/4 (1)	0/0/4 (2)			0/1/3 (2)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	0/0/4 (2)	0/0/4 (2)	1/3/0 (1)	1/0/3 (1)	0/0/4 (1)
<i>O. foliosus</i> Pérez Lara	4			0/0/4 (2)					0/0/4 (2)	0/0/4 (2)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	1/0/3 (1)				2/1/1 (3)*	
<i>O. hollitanus</i> (Lowe) Benth.	4	0/0/4 (2)	0/0/4 (1)	0/0/4 (2)	0/0/4 (1)	1/0/3 (1)			0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	0/1/3 (1)	0/0/4 (2)	1/0/3 (1)		0/0/4 (1)	
<i>O. kalifornis</i> (Pour. ex Willd.) Pau	4	0/0/4 (1)	0/0/4 (1)	3/1/0 (1)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	1/2/1 (1)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	0/1/3 (1)	0/0/4 (1)	0/0/4 (1)	0/1/3 (1)	0/0/4 (1)
<i>O. linkii</i> Heldr. & Sartori	4				0/1/3 (1)				3/1/0 (1)	0/0/4 (1)	0/0/4 (1)	0/1/3 (1)	0/1/3 (1)	0/0/4 (2)	0/0/4 (2)	3/1/0 (1)		2/2/0 (1)*	
ex Boiss.																			
<i>O. luteus</i> (L.) Clairv.	4	2/2/0 (1)	0/0/4 (5)	0/0/4 (2)	0/0/4 (2)	0/0/4 (2)	0/0/4 (5)		0/1/3 (2)	0/0/4 (4)	2/0/2 (4)	0/0/4 (2)	0/0/4 (2)	0/0/4 (2)	0/0/4 (4)	0/0/4 (2)	0/0/4 (1)	0/0/4 (1)	0/1/3 (5)
<i>O. maroccanus</i> Bolliger	4	3/0/1 (1)							1/1/2 (2)	0/0/4 (1)	0/1/3 (1)	0/1/3 (1)	0/1/3 (1)	1/0/3 (1)	2/1/1 (1)			2/2/0 (2)*	
<i>O. powellii</i> Maire	4								3/1/0 (1)	0/0/4 (2)	0/0/4 (2)	0/0/4 (2)	0/0/4 (2)	0/2/2 (2)				1/3/0 (3)*	
<i>O. pyrenaicus</i> subsp. <i>ablitianus</i> P. Monts.	4	2/0/2 (1)	2/2/0 (1)		0/0/4 (1)	0/0/4 (2)	3/0/1 (2)		0/0/4 (2)	0/0/4 (2)	0/0/4 (2)	0/0/4 (2)	0/0/4 (1)	0/0/4 (1)	2/0/2 (1)			0/0/4 (2)	
<i>O. pyrenaicus</i> (Bubani)	4	3/0/1 (1)	2/0/2 (3)	1/1/2 (2)		0/0/4 (2)	0/1/3 (6)	1/0/3 (2)	2/2/0 (1)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	0/0/4 (2)	0/0/4 (2)	3/1/0 (1)			0/0/4 (2)	
Rothm. subsp. <i>pyrenaicus</i>																			
<i>O. recordonii</i> Burnat & Barbey	4	0/0/4 (3)	0/0/4 (4)	0/0/4 (4)	0/0/4 (2)	0/1/3 (2)	0/0/4 (4)	0/0/4 (1)	0/0/4 (2)	0/0/4 (3)	0/0/4 (4)	0/0/4 (1)	0/0/4 (1)	0/0/4 (1)	0/0/4 (2)	0/0/4 (1)	0/0/4 (1)	1/0/3 (1)	0/0/4 (1)

TABLE 3. Continued.

Species ^b	n	Ov-2	Ov-3 ^c	Ov-5	Ov-6	Ov-10	Ov-12 ^c	Ov-15	Ov-17	Ov-19	Ov-20	Ov-21	Ov-25	Ov-26 ^c	Ov-28	Ov-30	Ov-32 ^c	Ov-33	Ov-35
<i>O. viscosus</i> subsp. <i>asturicus</i> M. Lainz	2		1/1/0 (1)						1/0/1 (1)	0/0/2 (2)			0/0/2 (1)		1/1/0 (1)	0/2/0 (1)			
<i>O. viscosus</i> subsp. <i>australis</i> (Boiss.) Jahand. & Maire	2	1/0/1 (1)		1/0/1 (1)		1/0/1 (1)			0/2/0 (1)	0/0/2 (2)			0/0/2 (2)		1/0/1 (1)				
<i>O. viscosus</i> subsp. <i>granatensis</i> (Boiss.) Bolliger	2	1/1/0 (1)	1/0/1 (1)	1/0/1 (1)		0/0/2 (1)	1/0/1 (1)		0/0/2 (1)	1/0/1 (1)			0/0/2 (2)		1/1/0 (1)				
<i>O. viscosus</i> subsp. <i>lusitanicus</i> Bolliger	2	1/0/1 (1)	0/2/0 (1)			0/0/2 (1)	0/1/1 (2)		0/0/2 (1)	0/0/2 (1)			0/0/2 (1)						
<i>O. viscosus</i> (L.) Clairv. subsp. <i>viscosus</i>	2	1/0/1 (1)	0/1/1 (2)			0/0/2 (1)	0/0/2 (1)		0/0/2 (1)	0/0/2 (1)			0/0/2 (1)		1/0/1 (1)				
<i>Pa. latifolia</i> (L.) Caruel	2	1/0/1 (1)		1/0/1 (1)						0/0/2 (2)			0/0/2 (1)						
<i>Pa. viscosa</i> (L.) Caruel	2	1/0/1 (2)		1/1/0 (1)*	0/2/0 (1)				0/1/1 (1)	0/0/2 (1)	0/0/2 (2)	0/0/2 (2)	0/0/2 (2)				1/0/1 (1)*		

Note: n = number of individuals sampled.

^a Amplification success is presented as: number of individuals that did not amplify/number of individuals that amplified weakly/number of individuals that amplified successfully (number of alleles detected). No amplification = peak height >0 and <250 relative fluorescence units (RFU); weak amplification = peak height >250 and <1000 RFU; successful amplification = peak height >1000 RFU; * = presence of spurious peak. Empty cells indicate failed amplification in all individuals.

^b Abbreviations: *B.* = *Barbisia*; *Ba.* = *Bellaradia*; *Eu.* = *Euphrasia*; *Ma.* = *Macrosyringium*; *No.* = *Nothobartsia*; *Od.* = *Odontitella*; *O.* = *Odontites*; *Pa.* = *Parentucellia*.

^c Loci excluded in *Odontites vernus* due to genotyping difficulties or no polymorphism.

APPENDIX 1. Voucher information for *Odontites* and related genera samples used in this study.

Species	Collector no. and voucher accession ^{a,b}	<i>n</i> ^c	Collection locality	Coordinates ^d
<i>Bartsia inaequalis</i> Benth.	S. Pfanzelt 999, SALA 153256	1	Bolivia: La Paz, Takesi valley	19KFB2480
<i>Bartsiaella rameauana</i> (Emb.) Bolliger	AQ 2129, MA 746138	2	Morocco: Azilal, Jbel Tarkeddit	29RQQ3692
<i>Bartsiaella rameauana</i>	VL 172, SALA 149231	2	Morocco: Ouarzazate, Tizi n' Ait Hamad	29RQQ5992
<i>Bellardia trixago</i> (L.) All.	DP 918, SALA 142076	1	Spain: Burgos, Castrillo de la Vega	30TVM3411
<i>Bellardia trixago</i>	MO 6020, SALA 142078	1	Spain: Cáceres, Gabriel y Galán Reservoir	29TQE4456
<i>Euphrasia antarctica</i> Benth.	S. Pfanzelt 699, CONC 180033	1	Chile: Magallanes, San Juan	19FCA7056
<i>Euphrasia hirtella</i> Jord. ex Reut.	ER 8041, SALA 142118	1	Spain: Ávila, San Martín de la Vega del Alberche	30TUK1778
<i>Macrosyringion longiflorum</i> (Lam.) Rothm.	DP 11, SALA 135639	1	Spain: Burgos, Castrillo de la Vega	30TVM3508
<i>Macrosyringion longiflorum</i>	DP 851, SALA 137313	1	Spain: Soria, Aldehuela de Periañez	30TWM5429
<i>Macrosyringion longiflorum</i>	DP 898, SALA 137290	1	Spain: Segovia, Ayllón	30TVL8073
<i>Macrosyringion longiflorum</i>	VL 82, SALA 137638	1	Morocco: Chefchaouen, Jbel L'akraa	30SUD0490
<i>Nothobartsia asperrima</i> (Link) Benedí & Herrero	DP 1062, SALA 156176	1	Morocco: Chefchaouen, track betw. Sidi Jel and Beni Bouker	30SUD0696
<i>Nothobartsia asperrima</i>	ER 7909, SALA 123313	1	Portugal: Ribatejo, Tomar	29SND4983
<i>Nothobartsia asperrima</i>	MS 958, SALA 123310	1	Portugal: Estremadura, Azeitão	29SNC0164
<i>Nothobartsia asperrima</i>	MS 960, SALA 123311	1	Portugal: Estremadura, Sesimbra	29SMC8151
<i>Nothobartsia spicata</i> (Ramond) Bolliger & Molau	ER 7920, SALA 125802	2	Spain: Oviedo, Ribadesella	30TUP3611
<i>Nothobartsia spicata</i>	ER 7921, SALA 125801	2	Spain: Santander, Peñarrubia	30TUN6791
<i>Odontitella virgata</i> (Link) Rothm.	DP 14, SALA 135636	1	Spain: Burgos, Castrillo de la Vega	30TVM3610
<i>Odontitella virgata</i>	ER 7959, SALA 136278	1	Spain: Cádiz, Los Barrios	30STF6712
<i>Odontitella virgata</i>	LD 1069, SALA 136280	1	Spain: A Coruña, Santiso	29TNH8046
<i>Odontitella virgata</i>	SA 297, SALA 135467	1	Portugal: Beira Litoral, rd. betw. Mira and Castanhede	29TNE2771
<i>Odontites bocconii</i> (Guss.) Walp.	G. Domina s.n., PAL 90581	2	Italy: Sicilia, San Martino delle Scale	33SUC4716
<i>Odontites bocconii</i>	JPG-11-03, SALA 142125	2	Italy: Sicilia, Madonie Regional Natural Park	33SVB1389
<i>Odontites bolligeri</i> E. Rico, L. Delgado & Herrero	AQ 2812, SALA 142142	1	Morocco: Berkane, Béni-Snassen	30SWD5652
<i>Odontites bolligeri</i>	DP 832, SALA 136804	1	Spain: Málaga, Frigiliana	30SVF1970
<i>Odontites bolligeri</i>	MO 4566, SALA 135619	1	Spain: Granada, Restábal	30SVF4886
<i>Odontites bolligeri</i>	VL 153, SALA 156172	1	Spain: Almería, Láujar de Andarax	30SWF1094
<i>Odontites cebennensis</i> H. J. Coste & Soulié	DP 628, SALA 135679	1	Spain: Barcelona, La Pobra de Lillet	31TDG1877
<i>Odontites cebennensis</i>	DP 1760, SALA 156184	1	Andorra: Ordino, track to Castell dels Moros	31TCH8012
<i>Odontites cebennensis</i>	DP 1842, SALA 156185	1	Spain: Gerona, Albanyà	31TDG7578
<i>Odontites cebennensis</i>	DP 1894, SALA 156186	1	Spain: Teruel, Linares de Mora	30TYK0665
<i>Odontites corsicus</i> (Loisel.) G. Don	A. Tribsch s.n., SALA 137639	4	France: Corse, Bastia	32TNN3133
<i>Odontites foliosus</i> Pérez Lara	DP 821, SALA 156297	1	Spain: Málaga, Manilva	30STF9724
<i>Odontites foliosus</i>	ER 7903, SALA 103775 ^e	1	Spain: Cádiz, Barbate	30STF3408
<i>Odontites foliosus</i>	ER 7939, SALA 134536	1	Spain: Cádiz, Puerto Real	29SQA5645
<i>Odontites foliosus</i>	VL 135, SALA 144130	1	Spain: Málaga, Genalguacil	30STF9947
<i>Odontites hollianus</i> (Lowe) Benth.	SC 17379, MA 714540	1	Portugal: Madeira, betw. Pico do Arreiro and Pico Ruivo	28SCB1823
<i>Odontites hollianus</i>	M. Díaz s.n., SALA 156496	2	Spain: Santa Cruz de Tenerife, Isla de La Palma	28RBS1482
<i>Odontites hollianus</i>	MS 5056, SALA 125030	1	Portugal: Madeira, betw. O Ninho da Manta and O Pico Cidrao	28SCB1724
<i>Odontites kaliformis</i> (Pourr. ex Willd.) Pau	ER 7913, SALA 124706	2	Spain: Valencia, Sagunto	30SYJ3690
<i>Odontites kaliformis</i>	ER 7914, SALA 124707	2	Spain: Castellón, Cabanes	31TBE6052
<i>Odontites linkii</i> Heldr. & Sartori ex Boiss.	AH 3359, SALA 140386	1	Greece: Peloponnese, Ahaia	34SFH0215
<i>Odontites linkii</i>	AH 3480, SALA 140486	1	Greece: Peloponnese, Korinthia	34SFH2804
<i>Odontites linkii</i>	CA 14257, SALA 140800	2	Greece: Peloponnese, Lakonia	34SFG1806
<i>Odontites luteus</i> (L.) Clairv.	BR 187, SALA 142123	1	Czech Republic: Jihomoravský kraj, betw. Klentnice and Mikulov	33UXQ2010
<i>Odontites luteus</i>	DP 763, SALA 137330	1	Spain: Albacete, Riópar	30SWH5361
<i>Odontites luteus</i>	DP 1018, SALA 110042	1	Spain: Valladolid, Santibañez de Valcorba	30TUM7904
<i>Odontites luteus</i>	ER 7852, SALA 136275	1	Spain: Lérida, betw. Puente de Montañana and Tremp	31TCG1670
<i>Odontites maroccanus</i> Bolliger	DP 785, SALA 156299	1	Morocco: Ifrane, Tizi-n-Tretten	30SUC1003
<i>Odontites maroccanus</i>	DP 1082, SALA 156177	1	Morocco: Ifrane, Ain Vittel	30SUC0314
<i>Odontites maroccanus</i>	DP 1084, SALA 156178	1	Morocco: Ifrane, Michlifen	30SUB0699

APPENDIX 1. Continued.

Species	Collector no. and voucher accession ^{a,b}	<i>n</i> ^c	Collection locality	Coordinates ^d
<i>Odontites maroccanus</i>	NLG 56, SALA 156170	1	Morocco: Ifrane, near Michlifen	30SUB0498
<i>Odontites powellii</i> Maire	AQ 2119, MA 746128	1	Morocco: Béni-Mellal, Tizzi-n-Aif	29SQS8002
<i>Odontites powellii</i>	DP 786, SALA 156298	1	Morocco: Ifrane, Tizi-n-Tretten	30SUC1003
<i>Odontites powellii</i>	NLG 64, SALA 156171	1	Morocco: Khénifra, Col du Zad	30SUB0750
<i>Odontites powellii</i>	VL 83, SALA 156300	1	Morocco: Chefchaouen, Jbel L'akraa	30SUD0490
<i>Odontites pyrenaicus</i> subsp. <i>abilianus</i> P. Monts.	DP 1603, SALA 156179	1	Spain: Huesca, Jaca	30TXN9312
<i>Odontites pyrenaicus</i> subsp. <i>abilianus</i>	DP 1607, SALA 156180	1	Spain: Zaragoza, Longás	30TXN6905
<i>Odontites pyrenaicus</i> subsp. <i>abilianus</i>	DP 1615, SALA 156181	1	Spain: Huesca, Jaca	30TYN0614
<i>Odontites pyrenaicus</i> subsp. <i>abilianus</i>	ER 7746, SALA 103068	1	Spain: Huesca, Jaca	30TXN9707
<i>Odontites pyrenaicus</i> (Bubani) Rothm. subsp. <i>pyrenaicus</i>	DP 615, SALA 135664	1	Spain: Lérida, Sarroca de Bellera	31TCG2492
<i>Odontites pyrenaicus</i> subsp. <i>pyrenaicus</i>	DP 1667, SALA 156182	1	Spain: Huesca, Isábena	31TCG0387
<i>Odontites pyrenaicus</i> subsp. <i>pyrenaicus</i>	DP 1736, SALA 156183	1	Spain: Lérida, Cabó	31TCG5375
<i>Odontites pyrenaicus</i> subsp. <i>pyrenaicus</i>	ER 7845, SALA 136276	1	Spain: Huesca, Plan	31TBH7515
<i>Odontites recordonii</i> Burnat & Barbey	DP 607, SALA 135656	1	Spain: Vitoria, Elciego	30TWN3008
<i>Odontites recordonii</i>	DP 672, SALA 135722	1	Spain: Albacete, Socovos	30SWH9242
<i>Odontites recordonii</i>	DP 692, SALA 135742	1	Spain: Guadalajara, Fuentelviejo	30TWK0184
<i>Odontites recordonii</i>	LD 1019, SALA 135629	1	Spain: Lérida, Sanatija	31TCG6136
<i>Odontites vernus</i> (Bellardi) Dumort.	A. Tribsch 4650, SALA 126029	1	Austria: Land Salzburg, Salzburg	33TUN5199
<i>Odontites vernus</i>	BR 27, SALA 135614	2	Bulgaria: Veliko Tarnovo, betw. Dobre Dyal and Rodina	35TMH0972
<i>Odontites vernus</i>	BR 127, SALA 137352	1	Serbia: Moravica, Čačak	34TDP3960
<i>Odontites vernus</i>	BR 158, SALA 142120	2	France: Haute-Normandie, near St. Sebastien	31UCQ6131
<i>Odontites vernus</i>	DP 619, SALA 135668	1	Spain: Lérida, Espot	31TCH4215
<i>Odontites vernus</i>	DP 636, SALA 135687	2 (1 ^{2x})	Spain: Gerona, Ribes de Freser	31TDG3181
<i>Odontites vernus</i>	DP 638, SALA 135689	3	Spain: Gerona, Campdevanol	31TDG3176
<i>Odontites vernus</i>	DP 663, SALA 135713	3	Spain: Granada, Quéntar	30SVG6420
<i>Odontites vernus</i>	DP 683, SALA 135733	2	Spain: Teruel, Linares de Mora	30TYK0465
<i>Odontites vernus</i>	DP 694, SALA 135744	2	Spain: Valladolid, Aldeamayor de San Martín	30TUL5997
<i>Odontites vernus</i>	DP 696, SALA 135746	32D+36T (1 ^{2x} +1 ^{4x})	Spain: Valladolid, San Miguel del Arroyo	30TUL7888
<i>Odontites vernus</i>	DP 999, SALA 110023	1	Spain: Burgos, Contreras	30TVM6352
<i>Odontites vernus</i>	DP 1277, SALA 150522	30	Spain: Burgos, Tejada	30TVM5544
<i>Odontites vernus</i>	ER 7844, SALA 110695	1 ^{4x}	Spain: Huesca, Saravillo	31TBH7415
<i>Odontites vernus</i>	ER 7851, SALA 110696	3	Spain: Huesca, Bisaurri	31TBH9509
<i>Odontites vernus</i>	ER 7863, SALA 110693	2	Spain: Toledo, Tembleque	30SVJ4592
<i>Odontites vernus</i>	ER 7876, SALA 110709	1 ^{2x}	Spain: Almería, Fondón	30SWF1293
<i>Odontites vernus</i>	ER 7890, SALA 110730	2	Spain: Lugo, Samos	29TPH4631
<i>Odontites vernus</i>	ER 7971, SALA 135644	1	Spain: Orense, Castro Caldelas	29TPG3089
<i>Odontites vernus</i>	ER 8053, SALA 156498	1	Spain: Burgos, Encío	30TVN9224
<i>Odontites vernus</i>	G. Domina s.n., PAL 88463	1 ^{2x}	Italy: Sicilia, Geraci Siculo	33SVB2592
<i>Odontites vernus</i>	G. Tuleu s.n., CBFS 5135	2 ^f	Czech Republic: South Bohemia, České Budějovice	33UVQ5925
<i>Odontites vernus</i>	LD 908, SALA 110700 ^c	2	Spain: Valladolid, Aldeamayor de San Martín	30TUL6698
<i>Odontites vernus</i>	LD 910, SALA 110698	3	Spain: Valladolid, Canillas de Esgueva	30TVM0723
<i>Odontites vernus</i>	LD 931, SALA 110715	1	Spain: Soria, El Royo	30TWM3235
<i>Odontites vernus</i>	LD 944, SALA 110736	2	Spain: Burgos, Oña	30TVN8228
<i>Odontites vernus</i>	LD 979, SALA 110715 ^c	2	Spain: Soria, El Royo	30TWM3235
<i>Odontites vernus</i>	MO 4522, SALA 135623	1 ^{4x}	Spain: Burgos, Merindad de Río Ubierna	30TVN4205
<i>Odontites vernus</i>	MO 5531, SALA 137348	2	Croatia: Lika-Senj, Plitvička Jezera National Park	33TWK5466
<i>Odontites vernus</i>	MO 5574, SALA 153253	1	Spain: Burgos, Frías	30TVN7635
<i>Odontites vernus</i>	MS 944, SALA 128791	1	Spain: Huesca, Ansó	30TXN8152
<i>Odontites vernus</i>	SA 415, SALA 137353	1	Macedonia: Kavadarci, betw. Rožden and Majden	34TEL7959
<i>Odontites viscosus</i> subsp. <i>asturicus</i> M. Lafnz	DP 874, SALA 137373	2	Spain: León, Puebla de Lillo	30TUN0774
<i>Odontites viscosus</i> subsp. <i>australis</i> (Boiss.) Jahand. & Maire	DP 566, SALA 136267	1	Spain: Granada, Güéjar Sierra	30SVG5712
<i>Odontites viscosus</i> subsp. <i>australis</i>	VL 91, SALA 156301	1	Morocco: Chefchaouen, Jbel L'akraa	30SUD0490
<i>Odontites viscosus</i> subsp. <i>granatensis</i> (Boiss.) Bolliger	JPG ODOGRA-G01, SALA 135386 ^c	1	Spain: Granada, Sierra Nevada	30SVG6207

APPENDIX 1. Continued.

Species	Collector no. and voucher accession ^{a,b}	<i>n</i> ^c	Collection locality	Coordinates ^d
<i>Odontites viscosus</i> subsp. <i>granatensis</i>	JPG 130, no voucher	1	Spain: Granada, Sierra Nevada	30SVG6208
<i>Odontites viscosus</i> subsp. <i>lusitanicus</i> Bolliger	MS 959, SALA 123308	1	Portugal: Estremadura, Sesimbra	29SMC8151
<i>Odontites viscosus</i> subsp. <i>lusitanicus</i>	MS 961, SALA 123309	1	Portugal: Estremadura, Sesimbra	29SMC8352
<i>Odontites viscosus</i> (L.) Clairv. subsp. <i>viscosus</i>	BR 165, SALA 142122	1	France: Provence-Alpes-Côte d'Azur, Marseille	31TFJ9705
<i>Odontites viscosus</i> subsp. <i>viscosus</i>	DP 616, SALA 135665	1	Spain: Lérida, Sarroca de Bellera	31TCG2492
<i>Parentucellia latifolia</i> (L.) Caruel	MO 6019, SALA 142077	2	Spain: Cáceres, Hervás	30TTK5659
<i>Parentucellia viscosa</i> (L.) Caruel	MO 6021, SALA 142079	2	Spain: Cáceres, betw. Guijo de Granadilla and Mohedas de Granadilla	29TQE3956

Note: *n* = number of individuals sampled.

^aAbbreviations (collector numbers): AH = Alberto Herrero; AQ =Alejandro Quintanar; BR = Blanca Rojas-Andrés; CA = Carlos Aedo; DP = Daniel Pinto; ER = Enrique Rico; JPG = Julio Peñas de Giles; LD = Luis Delgado; MO = M. Montserrat Martínez-Ortega; MS = María Santos (except for MS 5056, SALA 125030, which refers to Miguel Sequeira); NLG = Noemí López González; SA = Santiago Andrés-Sánchez; SC = Santiago Castroviejo; VL = Victor Lucía.

^bHerbarium specimens are lodged at the herbarium of Universidad de Salamanca (SALA), Salamanca, Spain; University of South Bohemia (CBFS), České Budějovice, Czech Republic; Universidad de Concepción (CONC), Concepción, Chile; Herbarium Mediterraneum Panormitanum (PAL), Palermo, Italy; and Real Jardín Botánico–Consejo Superior de Investigaciones Científicas (MA), Madrid, Spain. DNA samples are deposited at Biobanco de ADN Vegetal (Universidad de Salamanca), Salamanca, Spain.

^c2x, 4x indicate ploidy level of individuals used in initial screening by agarose gel electrophoresis.

^dCoordinates are in MGRS format and using WGS84 Datum.

^eSilica gel–dried material and voucher specimen were collected in the same location but on different dates.

^fIndividuals used to obtain 454 sequence library.

APPENDIX 2. Primers rejected and reasons for discarding.

Locus	Primer sequences (5'–3')	Repeat motif	PCR product size (bp)	T_a (°C)	GenBank accession no.	Discarding reason
Ov-1	F: TCCTTAGAAGGACCCCTCGAAAT R: TCAGTACATTTGTTACTTTTCAGCTA	(AAT) ₁₁	93	—	KT777565	Inconsistent amplification
Ov-3	F: CTCTCCTTCATCACCCTTCTT R: ACAAATTGAGAACCACCTTTCCC	(AC) ₁₁	124	54	KT777575	Genotyping difficulties
Ov-4	F: CACCTTTCTCATGAATCCATCT R: GTATGATGAAAAATGGACGGGTT	(AAAT) ₉	276	—	KT777576	Spurious bands in gel
Ov-7	F: GTCCGAAGCTCAAAGAGAAATC R: ACGTGAATAGATCTTCGACGGA	(CCG) ₇	81	—	KT777584	Low levels of polymorphism in gel
Ov-8	F: TGCCGTTAAAGTCTCAGATCAA R: ATAATTTCACTAACGGCGAAGC	(AC) ₁₀	103	—	KT777585	Low levels of polymorphism in gel
Ov-9	F: AATTCATAAGGCTGCTGCAGAT R: AATATCCATATGGTTTCAGCGG	(AG) ₁₀	84	—	KT777586	Low levels of polymorphism in gel
Ov-11	F: GATTCATGATTCGTTTATGTGT R: AATGCCACAACCTTTCATCTAA	(AAC) ₅	99	—	KT777591	Low levels of polymorphism in gel
Ov-12	F: AAAGATCTGCAACAAACAGCA R: GCATTATTCTCTATCCCACCCA	(AC) ₁₃	105	55	KT777592	Genotyping difficulties
Ov-13	F: TAAGCATAAAACCTGGAGGGGTC R: CGTTTGTGCGAGCTTTATTTTCC	(AC) ₁₀	108	—	—	Unsuccessful amplification
Ov-14	F: GCCACGATGTTTAGCCTTGTA R: GCTTCTCTTTTGTGGGGTTTATT	(AAT) ₆	161	—	—	Unsuccessful amplification
Ov-16	F: AGCTACCAATATTCAGGGGAT R: ATGGAATACTCCTCCCTCCCT	(AG) ₈	361	—	—	Unsuccessful amplification
Ov-18	F: CGTTCATCAACTTGACAAGAGC R: CAGAAGACCAACCACTCTCCT	(AG) ₂₂	179	—	—	Unsuccessful amplification
Ov-22	F: CAATTTAGGTGCAACTTGACACA R: GATATTCAGAATGACGGGAAGC	(ACC) ₅	159	—	KT777614	Spurious bands in gel
Ov-23	F: ACTCCTTTCGTTGCCTATACCA R: AGATGTCGTACTCGCAAACAGT	(AAT) ₅	82	—	KT777615	Low levels of polymorphism in gel
Ov-24	F: AGTTTTAGCTCCACAGTTGT R: CTTGAAATTGGTCTGGAAAGG	(ACC) ₅	89	—	KT777616	Low levels of polymorphism in gel
Ov-26	F: AAGGAGCTGATGAAAGCAGTTT R: AGCTCATATTCTCCGGGTACA	(AC) ₅	170	55	KT777621	Monomorphic
Ov-27	F: CTCAGTGTAGTTCGGTCAATGC R: GCAATTCACAAATTCATCCAA	(AG) ₆	276	—	—	Unsuccessful amplification
Ov-29	F: GTACCATATTTTCCACCACG R: ATGGAATACTCCTCCCTCCCT	(AG) ₈	275	—	—	Unsuccessful amplification
Ov-31	F: TGGGAGTAGGGTAATCAAAGGA R: AGAAGACCAACCAACTCTCCTG	(AG) ₂₂	225	—	—	Unsuccessful amplification
Ov-32	F: GATCCATTAGCAATGGGACTTT R: TCGAGGAGATGTAATGGTTTTG	(AG) ₁₁	411	53	KT777630	Genotyping difficulties
Ov-34	F: CGCATTTACGAATCAAACTAA R: AGCCTTGTAGCAGAAGCATTTT	(AC) ₅	208	—	—	Unsuccessful amplification
Ov-36	F: AATTCATCCTAGCGTGTCCAT R: ACTTGGTTGGGATACGTTTAGC	(AT) ₅	338	—	—	Unsuccessful amplification

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