Development of 14 Microsatellite Markers in Odontites vernus s.l. (Orobanchaceae) and Cross-Amplification in Related Taxa

Authors: Daniel Pinto-Carrasco, Jiří Košnar, Noemí López-González, Petr Koutecký, Jakub Těšitel, et. al.

Source: Applications in Plant Sciences, 4(3)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1500111

Applications in Plant Sciences 2016 4(3): 1500111

Primer Note

Development of 14 microsatellite markers in *Odontites vernus* s.l. (Orobanchaceae) and cross-amplification in related taxa[1]

Daniel Pinto-Carrasco[2,3,5], Jiří Košnar[4], Noemí López-González[2,3], Petr Koutecký[4], Jakub Těšitel[4], Enrique Rico[2,3], and M. Montserrat Martínez-Ortega[2,3]

[2]Departamento de Botánica, Universidad de Salamanca, E-37007 Salamanca, Spain; [3]Biobanco de ADN Vegetal, Banco Nacional de ADN, Edificio Multiusos I+D+I, E-37007 Salamanca, Spain; and [4]Faculty of Science, University of South Bohemia, CZ-370 05 České Budějovice, Czech Republic

- *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 Rhinantheae).
- *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; Rhinantheae.

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 Llueca, 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 (Invitek, 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 (Meglécz 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

[1]Manuscript received 29 September 2015; revision accepted 10 November 2015.

This research was financially supported by the Spanish Ministry of Science and Innovation through the projects CGL2011-28613-C03-03 and CGL2012-32574 and by the Czech Science Foundation through the project P505/12/1390. A predoctoral grant to D.P.C. from the Ministry of Education, Culture and Sport (AP2008-03528) is acknowledged. We are also deeply grateful to our laboratory technician, Teresa Malvar, for her support in the laboratory work.

[5]Author for correspondence: dpintocarrasco@usal.es

doi:10.3732/apps.1500111

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: CCCAAGTTTGTTAATTGGATCG R: GAACTGCAGCTGGAACCTCTA	VIC	$(AATT)_9$	171–213	11	Y	54	94	KT777566–KT777574
Ov-5	F: ATTAGGTACAACCACACGAGGG R: ATACTCGGCATCTTGCAATTCT	VIC	$(TA)_4$-$(CA)_8$	178–190	6	N	55	94	KT777577–KT777579
Ov-6	F: CACTCTCCCACGTTTCTTGATT R: TCAGAAATGGGGTATGAGAAAA	NED	$(AGC)_6$	92–116	13	Y	54	94	KT777580–KT777583
Ov-10	F: TGAATAATGTTTTCAGTCCATAC R: CACACTCTTGTAGCTATGTGCC	PET	$(AGT)_6$	213–217	4	Y	55	94	KT777587–KT777590
Ov-15	F: CTAGGGTTTGGGAATGTGGTT R: CCTAGCTACCCAGATACCATCC	NED	$(ACT)_5$	93–108	11	Y	54	*	KT777593–KT777596
Ov-17	F: TATCGATCCACTCGTGAAACAC R: TTCAGATCACGGTACACGATTC	PET	$(AAAAC)_5$	309–327	4	Y	55	94	KT777597–KT777599
Ov-19	F: GAGGAGGATTGAGGATTGCATA R: CCCACCATTTCATTTACTCTCC	5-FAM	$(AGGG)_5$	85–94	7	—	55	94	KT777600
Ov-20	F: GAGGAGACCCAATAACAAAATTA R: AATTTAAGCACCATGTTGAA	PET	$(AGTT)_3$	98–122	5	Y	55	83	KT777601–KT777603
Ov-21	F: GATCCATTAGCAATGGGACTTT R: TCCAGGTCAAACAGTGAACAAC	NED	$(AG)_{11}$	238–274	13	N	55	94	KT777604–KT777613
Ov-25	F: CTGCCATAGATAACATGCCAAA R: CCCCATGCCGAGAAAG	5-FAM	$(TA)_3$-$(CA)_9$	185–196	8	Y	55	94	KT777617–KT777620
Ov-28	F: ACAAGATTCTTCCCTCCCTGTC R: ATCCATGTGAGCAATGATGAAA	5-FAM	$(AC)_6$	227–258	8	Y	55	*	KT777622–KT777626
Ov-30	F: TTCGGATATCGAATAAAATGGG R: TCGCAATGTTTCCTGTTGTTAC	NED	$(AC)_5$	249–257	6	Y	55	94	KT777627–KT777629
Ov-33	F: CCTTGAACATACAGAAACTACAACAA R: TGATTACTATGCAACCACTGCC	VIC	$(AC)_5$	367–387	3	Y	55	94	KT777631–KT777633
Ov-35	F: TCAAATTCATTAGAACTGCGTCA R: CTATTTGACCATGAGCTCCACC	PET	$(ACC)_5$	310–313	2	N	55	83	KT777634–KT777635

Note: A = number of alleles; T_a = optimal annealing temperature; T_d = optimal denaturation temperature.
[a] Range of fragment sizes included the M13(–21) tail attached to the forward primer. Sizes were taken from GeneMarker allele calls.
[b] More 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.

[a] Deviations from HWE were statistically significant at *$P < 0.05$ and ***$P < 0.001$. Note that there were no deviations at $P < 0.01$.

[b] As 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 $MgCl_2$ (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′-TGTAAAACGACGGCCAGT-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* (Pourr. ex 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. pyrenaeus* (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.

LITERATURE CITED

Bennett, J. R., and S. Mathews. 2006. Phylogeny of the parasitic plant family Orobanchaceae inferred from phytochrome A. *American Journal of Botany* 93: 1039–1051.

Bolliger, M. 1996. Monographie der Gattung *Odontites* (Scrophulariaceae) sowie der verwandten Gattungen *Macrosyringion*, *Odontitella*, *Bornmuellerantha* und *Bartsiella. Willdenowia* 26: 37–168.

Delgado, L., D. Pinto Carrasco, F. Gallego Martín, and E. Rico. 2015. Contribution to the karyological knowledge of *Odontites* s.l. (Orobanchaceae) on the Iberian Peninsula and in Morocco. *Folia Geobotanica* 50: 63–74.

Excoffier, L., and H. E. L. Lischer. 2010. Arlequin suite version 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10: 564–567.

French, G. C., P. M. Hollingsworth, and R. A. Ennos. 2003. Isolation of polymorphic microsatellite markers for British *Euphrasia* L. *Molecular Ecology Notes* 3: 626–628.

Koutecký, P., G. Tuleu, T. Baďurová, J. Košnar, M. Štech, and J. Těšitel. 2012. Distribution of cytotypes and seasonal variation in the *Odontites vernus* group in central Europe. *Preslia* 84: 887–904.

López Udias, S., and C. Fábregat Llueca. 2010. *Odontites kaliformis* (Pourr. ex Willd.) Pau. *In* A. Bañares, G. Blanca, J. Güemes, J. C. Moreno, and S. Ortiz [eds.], Atlas y Libro Rojo de la Flora Vascular Amenazada de España, Adenda 2010, 84–85. Dirección General de Medio Natural y Política Forestal, Ministerio de Medio Ambiente y Medio Rural y Marino, Madrid, Spain.

Loureiro, J., E. Rodriguez, J. Doležel, and C. Santos. 2007. Two new nuclear isolation buffers for plant DNA flow cytometry: A test with 37 species. *Annals of Botany* 100: 875–888.

Meglécz, E., C. Costedoat, V. Dubut, A. Gilles, T. Malausa, N. Pech, and J. Martin. 2010. QDD: A user-friendly program to select microsatellite markers and design primers from large sequencing projects. *Bioinformatics (Oxford, England)* 26: 403–404.

Nilsson, L. A., and I. Alves-Dos-Santos. 2009. The oligolectic solitary bee *Melitta tricinta* Kirby, 1802 (Sw. rödtoppebi) in Sweden (Hymenoptera, Apoidea, Melittidae). *Entomologisk Tidskrift* 130: 85–98.

Nunome, T., S. Negoro, K. Miyatake, H. Yamaguchi, and H. Fukuoka. 2006. A protocol for the construction of microsatellite enriched genomic library. *Plant Molecular Biology Reporter* 24: 305–312.

Olejniczak, M., and W. J. Krzyzosiak. 2006. Genotyping of simple sequence repeats: Factors implicated in shadow band generation revisited. *Electrophoresis* 27: 3724–3734.

Parker, C. 2013. The parasitic weeds of the Orobanchaceae. *In* D. M. Joel, J. Gressel, and L. J. Musselman [eds.], Parasitic Orobanchaceae, 313–344. Springer, Berlin, Germany.

Rico, E. 2009. *Odontites* L. *In* C. Benedí, E. Rico, J. Güemes, and A. Herrero [eds.], Flora Iberica, vol. 13, 473–495. Real Jardín Botánico, Consejo Superior de Investigaciones Científicas, Madrid, Spain.

Schuelke, M. 2000. An economic method for the fluorescent labelling of PCR fragments. *Nature Biotechnology* 18: 233–234.

Snogerup, B. 1983. Northwest European taxa of *Odontites* (Scrophulariaceae). *Acta Botanica Fennica* 124: 1–62.

Temsch, E. M., J. Greilhuber, and R. Krisai. 2010. Genome size in liverworts. *Preslia* 82: 63–80.

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[c]	Ov-28	Ov-30	Ov-32[c]	Ov-33	Ov-35
B. inaequalis Benth.	1									0/0/1 (1)		0/0/1 (1)	0/0/1 (1)			0/0/1 (1)			
Ba. rameauana (Emb.) Bolliger	4		2/1/1 (2)	3/0/1 (1)			1/0/3 (4)			0/0/4 (2)			0/0/4 (1)		0/0/4 (3)			1/3/0 (2)*	
Be. trixago (L.) All.	2									0/0/2 (1)		0/0/2 (1)	0/0/2 (2)						
Eu. antarctica Benth.	1									0/0/1 (1)			0/1/0 (1)						
Eu. hirtella Jord. ex Reut.	1		0/0/1 (1)							0/0/1 (1)		0/0/1 (1)	0/1/0 (1)						
Ma. longiflorum (Lam.) Rothm.	4	3/0/1 (1)	3/0/1 (1)	0/0/4 (1)			3/0/1 (2)			0/2/2 (1)			0/0/4 (2)		0/1/3 (1)*	3/0/1 (1)		3/0/1 (1)*	
No. asperrima (Link) Benedí & Herrero	4				0/0/4 (1)		1/0/3 (1)			0/0/4 (1)			0/0/4 (1)						2/2/0 (2)
No. spicata (Ramond) Bolliger & Molau	4		3/0/1 (1)	3/1/0 (1)			0/0/4 (1)			0/0/4 (1)			0/0/4 (1)						1/2/1 (1)
Od. virgata (Link) Rothm.	4		2/0/2 (2)				3/0/1 (1)			0/0/4 (1)			0/0/4 (2)		3/0/1 (1)				
O. bocconii (Guss.) Walp.	4	2/0/2 (1)	3/0/1 (1)			0/0/4 (3)*				0/0/4 (2)			0/0/4 (1)	2/2/0 (1)	0/0/4 (3)			1/2/1 (1)*	
O. bolligeri 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 (1)		0/2/2 (4)	2/2/0 (1)	3/1/0 (1)	0/0/4 (1)*	
O. cebennensis 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 (2)	0/3/1 (3)		0/0/4 (1)	
O. corsicus (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)		3/0/1 (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)
O. foliosus Pérez Lara	4					0/0/4 (2)				0/0/4 (2)			0/0/4 (1)		1/0/3 (1)			2/1/1 (3)*	
O. hollianus (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)	0/1/3 (1)	0/0/4 (2)	1/0/3 (1)		0/0/4 (1)	
O. kaliformis (Pourr. 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 (3)	1/2/1 (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)		0/1/3 (1)	0/0/4 (1)
O. linkii Heldr. & Sartori ex Boiss.	4					0/1/3 (1)			3/1/0 (1)	0/0/4 (1)			0/1/3 (1)		0/0/4 (2)	3/1/0 (1)		2/2/0 (1)*	
O. luteus (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 (4)	0/0/4 (2)		0/0/4 (1)	0/1/3 (5)
O. maroccanus Bolliger	4	3/0/1 (1)							1/1/2 (2)	0/0/4 (1)			0/1/3 (1)		1/0/3 (1)	2/1/1 (1)		2/2/0 (2)*	
O. powellii Maire	4								3/1/0 (1)	0/0/4 (2)			0/0/4 (2)		0/2/2 (2)			1/3/0 (3)*	
O. pyrenaeus subsp. *abilianus* 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 (1)		0/0/4 (1)	2/0/2 (1)		0/0/4 (2)	
O. pyrenaeus (Bubani) Rothm. subsp. *pyrenaeus*	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 (2)		0/0/4 (2)	3/1/0 (1)		0/0/4 (2)	
O. recordonii 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 (2)	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
O. viscosus subsp. *asturicus* M. Laínz	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)			
O. viscosus subsp. *australis* (Boiss.) Jahand. & Maire	2		1/0/1 (1)				1/0/1 (1)		0/2/0 (1)	0/0/2 (2)			0/0/2 (2)			1/0/1 (1)			
O. viscosus subsp. *granatensis* (Boiss.) Bolliger	2		1/1/0 (1)	1/0/1 (1)					0/0/2 (1)	1/0/1 (1)			0/0/2 (2)			1/1/0 (1)			
O. viscosus subsp. *lusitanicus* Bolliger	2		1/0/1 (1)	0/2/0 (1)			0/1/1 (2)		0/0/2 (1)	0/0/2 (1)			0/0/2 (1)						
O. viscosus (L.) Clairv. subsp. *viscosus*	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)			1/0/1 (1)			
Pa. latifolia (L.) Caruel	2		1/0/1 (1)		1/0/1 (1)					0/0/2 (2)			0/0/2 (1)						
Pa. viscosa (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)					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.* = *Bartsia*; *Ba.* = *Bartsiella*; *Be.* = *Bellardia*; *Eu.* = *Euphrasia*; *Ma.* = *Macrosyringion*; *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]	*n*[c]	Collection locality	Coordinates[d]
Bartsia inaequalis Benth.	S. Pfanzelt 999, SALA 153256	1	Bolivia: La Paz, Takesi valley	19KFB2480
Bartsiella rameauana (Emb.) Bolliger	AQ 2129, MA 746138	2	Morocco: Azilal, Jbel Tarkeddit	29RQQ3692
Bartsiella rameauana	VL 172, SALA 149231	2	Morocco: Ouarzazate, Tizi n'Ait Hamad	29RQQ5992
Bellardia trixago (L.) All.	DP 918, SALA 142076	1	Spain: Burgos, Castrillo de la Vega	30TVM3411
Bellardia trixago	MO 6020, SALA 142078	1	Spain: Cáceres, Gabriel y Galán Reservoir	29TQE4456
Euphrasia antarctica Benth.	S. Pfanzelt 699, CONC 180033	1	Chile: Magallanes, San Juán	19FCA7056
Euphrasia hirtella Jord. ex Reut.	ER 8041, SALA 142118	1	Spain: Ávila, San Martín de la Vega del Alberche	30TUK1778
Macrosyringion longiflorum (Lam.) Rothm.	DP 11, SALA 135639	1	Spain: Burgos, Castrillo de la Vega	30TVM3508
Macrosyringion longiflorum	DP 851, SALA 137313	1	Spain: Soria, Aldehuela de Periañez	30TWM5429
Macrosyringion longiflorum	DP 898, SALA 137290	1	Spain: Segovia, Ayllón	30TVL8073
Macrosyringion longiflorum	VL 82, SALA 137638	1	Morocco: Chefchaouen, Jbel L'akraa	30SUD0490
Nothobartsia asperrima (Link) Benedí & Herrero	DP 1062, SALA 156176	1	Morocco: Chefchaouen, track betw. Sidi Jel and Beni Bouker	30SUD0696
Nothobartsia asperrima	ER 7909, SALA 123313	1	Portugal: Ribatejo, Tomar	29SND4983
Nothobartsia asperrima	MS 958, SALA 123310	1	Portugal: Estremadura, Azeitão	29SNC0164
Nothobartsia asperrima	MS 960, SALA 123311	1	Portugal: Estremadura, Sesimbra	29SMC8151
Nothobartsia spicata (Ramond) Bolliger & Molau	ER 7920, SALA 125802	2	Spain: Oviedo, Ribadesella	30TUP3611
Nothobartsia spicata	ER 7921, SALA 125801	2	Spain: Santander, Peñarrubia	30TUN6791
Odontitella virgata (Link) Rothm.	DP 14, SALA 135636	1	Spain: Burgos, Castrillo de la Vega	30TVM3610
Odontitella virgata	ER 7959, SALA 136278	1	Spain: Cádiz, Los Barrios	30STF6712
Odontitella virgata	LD 1069, SALA 136280	1	Spain: A Coruña, Santiso	29TNH8046
Odontitella virgata	SA 297, SALA 135467	1	Portugal: Beira Litoral, rd. betw. Mira and Castanhede	29TNE2771
Odontites bocconii (Guss.) Walp.	G. Domina s.n., PAL 90581	2	Italy: Sicilia, San Martino delle Scale	33SUC4716
Odontites bocconii	JPG-11-03, SALA 142125	2	Italy: Sicilia, Madonie Regional Natural Park	33SVB1389
Odontites bolligeri E. Rico, L. Delgado & Herrero	AQ 2812, SALA 142142	1	Morocco: Berkane, Béni-Snassen	30SWD5652
Odontites bolligeri	DP 832, SALA 136804	1	Spain: Málaga, Frigiliana	30SVF1970
Odontites bolligeri	MO 4566, SALA 135619	1	Spain: Granada, Restábal	30SVF4886
Odontites bolligeri	VL 153, SALA 156172	1	Spain: Almería, Láujar de Andarax	30SWF1094
Odontites cebennensis H. J. Coste & Soulié	DP 628, SALA 135679	1	Spain: Barcelona, La Pobla de Lillet	31TDG1877
Odontites cebennensis	DP 1760, SALA 156184	1	Andorra: Ordino, track to Castell dels Moros	31TCH8012
Odontites cebennensis	DP 1842, SALA 156185	1	Spain: Gerona, Albanyà	31TDG7578
Odontites cebennensis	DP 1894, SALA 156186	1	Spain: Teruel, Linares de Mora	30TYK0665
Odontites corsicus (Loisel.) G. Don	A. Tribsch s.n., SALA 137639	4	France: Corse, Bastia	32TNN3133
Odontites foliosus Pérez Lara	DP 821, SALA 156297	1	Spain: Málaga, Manilva	30STF9724
Odontites foliosus	ER 7903, SALA 103775[e]	1	Spain: Cádiz, Barbate	30STF3408
Odontites foliosus	ER 7939, SALA 134536	1	Spain: Cadiz, Puerto Real	29SQA5645
Odontites foliosus	VL 135, SALA 144130	1	Spain: Málaga, Genalguacil	30STF9947
Odontites hollianus (Lowe) Benth.	SC 17379, MA 714540	1	Portugal: Madeira, betw. Pico do Arieiro and Pico Ruivo	28SCB1823
Odontites hollianus	M. Díaz s.n., SALA 156496	2	Spain: Santa Cruz de Tenerife, Isla de La Palma	28RBS1482
Odontites hollianus	MS 5056, SALA 125030	1	Portugal: Madeira, betw. O Ninho da Manta and O Pico Cidrão	28SCB1724
Odontites kaliformis (Pourr. ex Willd.) Pau	ER 7913, SALA 124706	2	Spain: Valencia, Sagunto	30SYJ3690
Odontites kaliformis	ER 7914, SALA 124707	2	Spain: Castellón, Cabanes	31TBE6052
Odontites linkii Heldr. & Sartori ex Boiss.	AH 3359, SALA 140386	1	Greece: Peloponnese, Ahaia	34SFH0215
Odontites linkii	AH 3480, SALA 140486	1	Greece: Peloponnese, Korinthia	34SFH2804
Odontites linkii	CA 14257, SALA 140800	2	Greece: Peloponnese, Lakonia	34SFG1806
Odontites luteus (L.) Clairv.	BR 187, SALA 142123	1	Czech Republic: Jihomoravský kraj, betw. Klentnice and Mikulov	33UXQ2010
Odontites luteus	DP 763, SALA 137330	1	Spain: Albacete, Riópar	30SWH5361
Odontites luteus	DP 1018, SALA 110042	1	Spain: Valladolid, Santibañez de Valcorba	30TUM7904
Odontites luteus	ER 7852, SALA 136275	1	Spain: Lérida, betw. Puente de Montañana and Tremp	31TCG1670
Odontites maroccanus Bolliger	DP 785, SALA 156299	1	Morocco: Ifrane, Tizi-n-Tretten	30SUC1003
Odontites maroccanus	DP 1082, SALA 156177	1	Morocco: Ifrane, Aïn Vittel	30SUC0314
Odontites maroccanus	DP 1084, SALA 156178	1	Morocco: Ifrane, Michlifen	30SUB0699

Appendix 1. Continued.

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

Appendix 1. Continued.

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

Note: n = number of individuals sampled.

[a]Abbreviations (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.

[b]Herbarium 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.

[c]$2x$, $4x$ indicate ploidy level of individuals used in initial screening by agarose gel electrophoresis.

[d]Coordinates are in MGRS format and using WGS84 Datum.

[e]Silica gel–dried material and voucher specimen were collected in the same location but on different dates.

[f]Individuals 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: TCCTTAGAAGGACCCTCGAAAT R: TCAGTACATTTGTTACTTTTCAGCTA	$(AAT)_{11}$	93	—	KT777565	Inconsistent amplification
Ov-3	F: CTCTCCTTCATCACCCCTTCTT R: ACAAATTGAGAACCACTTTCCC	$(AC)_{11}$	124	54	KT777575	Genotyping difficulties
Ov-4	F: CACCTTTCTCATGAATCCATCT R: GTATGATGAAAATGGACGGGTT	$(AAAT)_9$	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: GATTCATTGATTCGTTTATGTGT R: AATGCCACAACTTTGCATCTAA	$(AAC)_5$	99	—	KT777591	Low levels of polymorphism in gel
Ov-12	F: AAAGATCTGCAAACAAACAGCA R: GCATTATTCTCTATCCCACCCA	$(AC)_{13}$	105	55	KT777592	Genotyping difficulties
Ov-13	F: TAAGCATAAAACTGGAGGGGTC R: CGTTTGTCGAGCTTTATTTTCC	$(AC)_{10}$	108	—	—	Unsuccessful amplification
Ov-14	F: GCCACGTATGTTTAGCCTTGTA R: GCTTCTCTTTTGTGGGGTTTATT	$(AAT)_6$	161	—	—	Unsuccessful amplification
Ov-16	F: AGCTACCCAATATTCAGGGGAT R: ATGGAATACTCCTCCCTCCCT	$(AG)_8$	361	—	—	Unsuccessful amplification
Ov-18	F: CGTTCATCAACTTGACAAGAGC R: CAGAAGACCAACCAACTCTCCT	$(AG)_{22}$	179	—	—	Unsuccessful amplification
Ov-22	F: CAATTTAGGTCGAACTTGCACA 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: AGTTTTCAGCTCCACAGGTTGT R: CTTGAAATTGGTTCTGGAAAGG	$(ACC)_5$	89	—	KT777616	Low levels of polymorphism in gel
Ov-26	F: AAGGAGCTGATGAAAGCAGTTT R: AGCTCATATTCTCCGGGTTACA	$(AC)_5$	170	55	KT777621	Monomorphic
Ov-27	F: CTCAGTGTAGTTCCGTCATTGC R: GCAATTCACAAATTCAATCCAA	$(AG)_6$	276	—	—	Unsuccessful amplification
Ov-29	F: GTACCCATATTTTTCCACCACG 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: CGCATTTCACGAATCAAACTAA R: AGCCTTGTAGCAGAAGCATTTC	$(AC)_5$	208	—	—	Unsuccessful amplification
Ov-36	F: AATTCATCCTAGCGTGTTCCAT R: ACTTGGTTGGGATACGTTTAGC	$(AT)_5$	338	—	—	Unsuccessful amplification

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