

1 **Development and characterization of microsatellite loci for the perennial herb**

2 *Helleborus foetidus* (Ranunculaceae)

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23 **Abstract**

24 Microsatellite loci were developed for *Helleborus foetidus* by next-generation 454
25 pyrosequencing of a microsatellite-enriched library. We selected 60 primer pairs, from which
26 13 microsatellite markers exhibited polymorphism and 13 were monomorphic. The number of
27 alleles per locus ranged from two to eight (mean=3.92) and expected heterozygosity ranged
28 from 0.05 to 0.77. One locus showed heterozygote deficiency due to the presence of null
29 alleles, and no pairs of loci demonstrated linkage disequilibrium. The newly developed
30 markers will enable population genetic studies and address specific questions involving
31 mating system and pollen dispersal strategies in this species.

32

33 **Main text**

34 *Helleborus foetidus* L. (Ranunculaceae) is a diploid (Dhooghe *et al.* 2009) perennial
35 herb widely distributed across Western Europe that grows in the understory of deciduous and
36 mixed forests (Werner & Ebel 1994). Flowering takes place over winter and early spring.
37 Flowers are hermaphroditic, protogynous, self-compatible and extremely long lived (up to 20
38 days; Vesprini & Pacini 2000). *Helleborus foetidus* is mainly pollinated by bumblebees,
39 which forage for the abundant, sucrose-rich nectar this species offers (Vesprini *et al.* 1999).
40 The reproductive biology and ecology of *H. foetidus* have been thoroughly investigated (e.g.
41 Herrera *et al.* 2001; Rey *et al.* 2006 and references therein), but the lack of suitable molecular
42 markers have so far hampered crucial studies on genetic diversity and gene flow. The
43 microsatellite markers presented here will allow us to conduct specific research on how biotic
44 factors, such as the presence of nectar yeasts in floral nectar (see Herrera *et al.* 2008; Herrera
45 & Pozo 2010; Herrera *et al.* in press) may affect pollen dispersal strategies, mating pattern
46 and seed progeny quality in this species.

47

48 For isolation of microsatellite loci, a high-throughput method coupling microsatellite
49 DNA enrichment and 454 GS-FLX Titanium next-generation sequencing was carried out by
50 Genoscreen (Lille, France) as described by Malausa *et al.* (2011), with modifications
51 regarding oligonucleotide probes. Sequences of the oligonucleotide probes used were as
52 follows: TG, TC, AAC, AAG, AGG, ACG, ACAT, ACTC. The resulting sequences were
53 analysed according to the pipeline implemented in QDD (Méglec *et al.* 2010), yielding
54 primer sequences for 1010 potential microsatellite loci. We then selected 60 primer pairs for
55 amplification trials based on primer design and suitability of expected fragment sizes for
56 multiplexing purposes.

57 Total genomic DNA was extracted with Qiacube using DNeasy Plant Mini Kit
58 (Qiagen) following manufacturer's protocol, from 20-25 mg sample of dried leaf material
59 ground to fine powder in a Retsch MM 200 mill. Concentration and quality of DNA extracts
60 were estimated through spectrophotometry using Nanodrop (Thermo Scientific). For initial
61 simplex amplification tests we used 15 *H. foetidus* individuals collected from six natural
62 populations located in our study area in Sierra de Cazorla (Jaén province, southeastern Spain):
63 Coto del Valle, Castellón de los Cierzos, Rastrillos de la Víbora, Fuente Bermejo, La Cabrilla
64 and Roblehondo. Fluorescent labelling of PCR products in simplex PCR was done by
65 attaching a 'M13-tail' (5'-CAGTCGGGCGTCATCA- 3') to the 5' end of the forward primers,
66 following the method described by Schuelke (2000). Simplex PCR reactions were performed
67 in 20 μ L volume containing 1 \times BSA (New England Biolabs), 1 \times reaction buffer, 3.5 mM
68 $MgCl_2$, 0.5 U *Taq* DNA polymerase (Bioline), 0.25 mM dNTP (Sigma-Aldrich), 0.3 μ M of
69 each 'M13-tailed' forward primer, reverse primer and universal 'M13' primer tagged to the 5'
70 end with one of FAM, VIC, NED or PET (Applied Biosystems), and c.45 ng of DNA extract

71 as template. Simplex PCR conditions consisted of 95°C for 3 min, 19 cycles of 95°C for 1
72 min, 58°C for 30 sec of annealing with temperature decreasing 0.5°C every cycle, 72°C for 30
73 sec, followed by 19 cycles of 95°C for 1 min, 48°C for 30 sec and 72°C for 30 sec. A final
74 extension step consisted in 72°C for 10 min.

75 Primer pairs providing polymorphic, consistently scorable allele peaks obtained with
76 simplex PCR conditions were further tested in 60 individuals from 3 populations of the same
77 region (N = 20 individuals per population), distances between sites ranging from 5 to 11 km:
78 Tejerina (TEJ; 37.98° N, 2.91° W, 740 m.a.s.l.), Las Navillas (NAV; 37.94° N, 2.91° W, 1235
79 m.a.s.l.) and Puerto Llano (PLL; 37.81° N, 2.96° W, 1810 m.a.s.l.). Primers were
80 fluorescently labelled and conditions optimized to perform multiplex PCR including most of
81 the selected primers. Multiplex PCR profiles were optimized to combine 5 polymorphic
82 microsatellite markers each in a single 20 µL reaction containing 1× reaction buffer, 3.5 mM
83 MgCl₂, 0.25 mM dNTP, 2 U *Taq* DNA polymerase, 0.2 µM of 5'-labeled primer with one of
84 FAM, VIC, NED or PET tags, 0.2 µM unlabelled primer and c.120 ng of DNA extract as
85 template. Conditions for multiplex PCR were: 95°C for 3 min, 38 cycles of 95°C for 30 sec,
86 58°C for 30 sec, 72°C for 1 min and a final extension of 72°C for 3 min. Three additional
87 primer pairs were optimized with simplex PCR reactions as described above (see Table 1).
88 All PCR reactions were performed in a C100 Thermal Cycler (Biorad).

89 Diluted PCR products were separated and sized according to 500 LIZ size standard in
90 an ABI PRISM 3130xl sequencer (Applied Biosystems). Allele patterns based on peak
91 fluorescent intensity and morphology were visually analysed using GENEMAPPER v4.0
92 (Applied Biosystems). Total number of alleles, observed and expected heterozygosity and
93 private alleles (i.e. alleles detected in one population only) were computed with GENALEX 6
94 (Peakall & Smouse 2006). Frequency of null alleles was estimated with INEST 1.1 (Chybicki

95 & Burczyk 2009; Campagne *et al.* 2012). Deviation from Hardy-Weinberg equilibrium,
96 linkage disequilibrium between pairs of loci and fixation index (F_{IS} ; Weir & Cockerham
97 1984) were calculated with GENEPOP v4 (Rousset 2008).

98

99 Twenty-six out of the 60 tested loci produced clear amplification products. Thirteen
100 loci exhibited polymorphism (*Hefo1-Hefo13*) and enabled resolution of 60 individual
101 genotypes, whereas other 13 loci appeared to be monomorphic (*Hefo14-Hefo26*). GenBank
102 accession numbers, primer sequences, repeat motifs and PCR profiles for polymorphic loci
103 are listed in Table 1. The remaining 34 primer pairs produced unspecific peaks or failed to
104 amplify and therefore were dismissed.

105 Among polymorphic loci, the number of alleles per locus ranged from two to eight
106 (mean = 3.92) with a total of 51 alleles in the 60 genotyped individuals. The number of
107 private alleles in each population was two for TEJ, three for NAV and three for PLL.
108 Chybicki-Burczyk likelihood-based method after 10000 iterations indicated high null allele
109 frequency only at locus *Hefo12* in all populations (Table 2). All loci were at Hardy-Weinberg
110 equilibrium except *Hefo12*, which showed a significant excess of homozygotes, due to the
111 presence of null alleles. None of the loci pairs demonstrated linkage disequilibrium after
112 Bonferroni correction for multiple comparisons. Population mean F_{IS} values were 0.044 for
113 TEJ, 0.035 for NAV and 0.032 for PLL.

114 In conclusion, the microsatellite loci described here will be of great value for further
115 studies on mating system, as well as assessing patterns of gene flow and levels of genetic
116 diversity in natural populations of *H. foetidus*, and specifically, in the study of the interactions
117 of nectar-living microbes affecting pollen dispersal and seed progeny quality in this species.

118

119 ***Data accessibility***

120 GenBank accession numbers for *H. foetidus* microsatellite loci: JX905360 to JX905372
121 (polymorphic loci) and JX905373 to JX905385 (monomorphic loci).

122

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129 ***Contributors***

130 MMS and CdV carried out genetic analyses and were responsible for the writing and edition
131 of the manuscript. PB conducted most laboratory work. MM collected field samples used in
132 the present study and conducted some analyses, and CMH designed and supervised the study.

133

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Table 1. Characteristics of polymorphic loci developed for *Helleborus foetidus*. F= forward primer, R= reverse primer, M1=Multiplex set 1, M2=Multiplex set 2, S=Simplex and N_A= total number of alleles in the whole sample of 60 individuals from 3 populations (see Table 2). A 'PIGtail' (5'-GTTT-3') was added to the 5' end of reverse primers to facilitate the addition of adenosine by *Taq* polymerase (Brownstein *et al.* 1996).

Locus name	Accession number	Primer sequence (5'-3')	Repeat motif	Dye	PCR Profile	Size range (bp)	N _A
<i>Hefo1</i>	JX905360	F: TTGGAAATTTTAAAGGTTCTTGC R: CCAACCACATAATCATATCATAAGC	(TCT) ₇	FAM	M1	197-203	3
<i>Hefo2</i>	JX905361	F: AGCACTGAGTTTCTAAAAGGGC R: TTCTCTTCAATCAAGAATAAACCA	(GA) ₇	VIC	M1	95-97	2
<i>Hefo3</i>	JX905362	F: AACATGCAAGACCGAACAAC R: CCGGCGAAACTTTACCTGT	(GA) ₁₂	PET	M2	101-117	8
<i>Hefo4</i>	JX905363	F: CTCTTAAGTTTATCAGAACTTTGC R: AGTATCTAGGTGGATAACGCTTGA	(TCT) ₈	FAM	M1	247-265	7
<i>Hefo5</i>	JX905364	F: AACCACTCTATACGCTCCTCCA R: TTGAGATAGTAGCACCTATTATTGAGA	(CT) ₇	FAM	S	173-177	3
<i>Hefo6</i>	JX905365	F: ACTCACCAGTTTGGTTTTGCT R: TGCATACTCAATCCCATCCA	(GTT) ₅	FAM	M1	164-167	2
<i>Hefo7</i>	JX905366	F: CTTAACTGTACACCCTTAATGCATATC R: CAAGATACTCAAGCATGGGC	(CA) ₈	FAM	S	153-155	2
<i>Hefo8</i>	JX905367	F: GGAAGTACTCGAGGAAATTAACGA R: CCCAACTTATGATCTGCCAAA	(GA) ₉	NED	M2	202-218	3
<i>Hefo9</i>	JX905368	F: AACCATCCATTACACCATTT R: TTTGTATTGGCATTTCATGG	(CT) ₇	NED	M2	266-268	2
<i>Hefo10</i>	JX905369	F: AGCTTGCACAATGCTCTTCA	(GA) ₁₀	VIC	M1	174-178	3

<i>Hefo11</i>	JX905370	R: CGATGCAAGTTGGTTCTTTTC F: AGGTTCTAACCAAACCAATAAGG	(TC) ₈	FAM	S	265-269	3
<i>Hefo12</i>	JX905371	R: GGATTGATATGACTTCATCACTGG F: AGGGTAACGAAGGATATGACAGC	(CT) ₁₀	PET	M2	185-193	5
<i>Hefo13</i>	JX905372	R: TGGGTGTTAAGAAGGTAATAAGATG F: AATAGGCCACCAGGGTTGAT	(AG) ₈	NED	M2	162-176	8
		R: TCTTGATAGGCCTCATTATTTGT					

Table 2. Population genetics statistics based on the 13 polymorphic microsatellite loci in three *Helleborus foetidus* populations (TEJ, NAV, and PLL, N = 20 individuals) located at Sierra de Cazorla (southeastern Spain). N_A =Total number of alleles, H_O =observed heterozygosity, H_E =expected heterozygosity, F_{IS} =Weir and Cockerham's (1984) fixation index, HWE=significant deviation from Hardy-Weinberg equilibrium, r = null allele frequency estimated by Chybicki-Burczyk method and S.D.=standard deviation.

Locus	TEJ						NAV						PLL					
	N_A	H_O	H_E	F_{IS}	HWE ¹	r	N_A	H_O	H_E	F_{IS}	HWE ¹	r	N_A	H_O	H_E	F_{IS}	HWE ¹	r
<i>Hefo1</i>	3	0.400	0.483	0.176		0.093	3	0.450	0.504	0.109		0.074	2	0.450	0.450	-0.000		0.077
<i>Hefo2</i>	2	0.250	0.224	-0.118		0.079	2	0.350	0.409	0.147		0.098	2	0.100	0.097	-0.027		0.104
<i>Hefo3</i>	5	0.800	0.773	-0.036		0.042	4	0.650	0.682	0.048		0.060	6	0.500	0.587	0.152		0.075
<i>Hefo4</i>	7	0.750	0.681	-0.105		0.038	6	0.550	0.690	0.207		0.080	5	0.400	0.574	0.309		0.110
<i>Hefo5</i>	2	0.400	0.328	-0.226		0.060	3	0.550	0.476	-0.161		0.048	2	0.350	0.409	0.147		0.098
<i>Hefo6</i>	2	0.500	0.385	-0.310		0.052	2	0.850	0.512	-0.691		0.030	2	0.400	0.328	-0.226		0.061
<i>Hefo7</i>	2	0.300	0.262	-0.152		0.071	2	0.050	0.050	n.a.		0.109	2	0.100	0.097	-0.027		0.104
<i>Hefo8</i>	2	0.400	0.431	0.073		0.086	3	0.350	0.465	0.253		0.106	2	0.200	0.185	-0.086		0.084
<i>Hefo9</i>	2	0.350	0.481	0.277		0.115	2	0.650	0.512	-0.280		0.048	2	0.600	0.508	-0.188		0.055
<i>Hefo10</i>	3	0.400	0.396	-0.010		0.075	3	0.200	0.344	0.424		0.142	2	0.100	0.097	-0.027		0.098
<i>Hefo11</i>	2	0.050	0.050	n.a. ²		0.112	3	0.550	0.528	-0.042		0.062	3	0.400	0.347	-0.156		0.061
<i>Hefo12</i>	5	0.105	0.724	0.858	***	0.356	4	0.313	0.615	0.500	*	0.171	4	0.400	0.671	0.410	*	0.155
<i>Hefo13</i>	5	0.500	0.555	0.102		0.070	6	0.700	0.641	-0.095		0.038	6	0.650	0.747	0.133		0.063
Average	3.23	0.400	0.444	0.044			3.31	0.478	0.494	0.035			3.08	0.358	0.392	0.032		
S.D.	1.69	0.214	0.207	0.306			1.38	0.221	0.168	0.323			1.61	0.184	0.224	0.190		

¹* ($p < 0.05$) and *** ($p < 0.001$). Empty cells indicate no deviation from HWE.

²n.a. = not available.