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PRIMER NOTE

DEVELOPMENT OF MICROSATELLITE MARKERS FOR *CREPIS MOLLIS* (ASTERACEAE)[1]

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- *Premise of the study:* Polymorphic microsatellite markers were developed for the threatened species *Crepis mollis* (Asteraceae) to investigate population and conservation genetics.
- *Methods and Results:* Illumina sequencing was conducted on pooled genomic DNA from 10 individuals of two populations. Ten polymorphic and 10 monomorphic microsatellite loci with di-, tri-, tetra-, penta-, and hexanucleotide repeat motifs were developed and characterized in *C. mollis*. In the polymorphic markers, up to 17 alleles per locus were detected with an observed and expected heterozygosity ranging from 0.120 to 0.780 and 0.102 to 0.834, respectively. Furthermore, the polymorphic markers were tested for cross-amplification in three congeneric species (*C. biennis*, *C. foetida*, and *C. sancta*) and amplified in up to three loci.
- *Conclusions:* The markers developed in this study are the first microsatellites tested on *C. mollis* and will be useful for performing population and conservation genetic studies in this threatened species.

Key words: Asteraceae; *Crepis mollis*; Illumina; microsatellites; population genetics.

Crepis mollis (Jacq.) Asch. is a short-lived perennial yellow herb in the Asteraceae and is distributed in temperate Europe ranging from the Ukraine, western Russia, and the Baltic states in the east to Italy, the Pyrenees, Great Britain, and Germany in the west; it is not found outside of Europe (Kilian et al., 2009; O'Reilly, 2010). The genus *Crepis* L. is thought to be insect pollinated and self-compatible, and the dispersal is by anemochory, epizoochory, or even myrmecochory (Bundesamt für Naturschutz, 2011). *Crepis mollis* colonizes meadows and pastures with a medium supply of water and nutrients, but also occurs in fens, near ponds, and in marshy banks. It can be found from the lowlands to the subalpine zone in the Alps up to an altitude of about 2000 m (Hegi, 1987; Bundesamt für Naturschutz, 2011). The abandonment of the extensive use of grassland, eutrophication, and the loss of extensively grazed wood pasture on base-rich soils have lead to a strong decline of this species in Central Europe (Meusel and Jäger, 1992; Braithwaite, 2004). *Crepis mollis* is not listed under the IUCN Red List, but is classified as "threatened" in the national assessments of vascular plants of Germany (Korneck et al., 1996).

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To develop prospective conservation strategies for *C. mollis*, it is necessary to understand the population genetic structure and genetic diversity of this declining and understudied species. Because very little is known about the genetic structure of *C. mollis* and no genetic markers have been developed for this species so far, we characterized a set of polymorphic microsatellite markers useful for population genetic investigations as the basis for scientifically informed conservation measures. Furthermore, we investigated cross-amplification in the congeneric taxa *C. biennis* L., *C. foetida* L., and *C. sancta* (L.) Babc. and their subspecies *C. foetida* subsp. *foetida*, *C. foetida* subsp. *communata* (Spreng.) Babc., and *C. sancta* subsp. *bifida* Thell. ex Babc.

METHODS AND RESULTS

***Plant material and DNA extraction*—**Plant material of *C. mollis* was collected in Germany from five populations between 14 and 400 km apart from each other (Erzgebirge, Saxony and the Alps, Bavaria). From each population, 10 individuals were sampled for leaf tissue, of which one individual was collected as a voucher specimen and deposited at the herbarium of the Botanical Garden and Botanical Museum Berlin-Dahlem (B). The leaf samples were dried with silica gel, and genomic DNA was extracted with the NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. The final concentration of 100 µL purified and eluted DNA was quantified using a NanoDrop ND-1000 spectrophotometer (Peqlab, Erlangen, Germany).

For testing cross-amplification, DNA samples of three congeneric species (*C. biennis* [*N* = 6], *C. foetida* [*N* = 6], and *C. sancta* [*N* = 9]) were provided by the DNA bank at B and are available via the Global Genome Biodiversity Network (GGBN, 2011). For each sample, the corresponding voucher specimen is deposited at B (Appendix 1).

***Microsatellite marker development*—**The Illumina Nextera DNA Sample Preparation Kit (Illumina, San Diego, California) and the Nextera Index Kit

TABLE 1. Characteristics of the 10 polymorphic and 10 monomorphic microsatellite loci developed for *Crepis mollis*.

Locus[a]	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	Fluorescent label[b]	GenBank accession no.
Polymorphic loci					
Cremo13	F: GGCTCTATAAGCGGCACAAC	$(AGG)_6$	246–249	Yakima Yellow	KT992812
	R: GCTTCACTTCCAGGAACAGG				
Cremo14	F: TTTGATGAAAGAGGGTTGAAAG	$(AAC)_{10}$	128–155	FAM	KT992813
	R: TTAGATAGCACTATGTGGAACCC				
Cremo15	F: GGGAACTCCAAATGTTAAGGC	$(AAT)_7$	116–119	ATTO 550	KT992814
	R: TTTGGTTAGTCATGATACACCTGC				
Cremo26	F: AGTGCTGATGCCTGTGTCTG	$(AG)_9$	180–216	ATTO 565	KT992815
	R: TGTGGACATGCTGAGGACAG				
Cremo33	F: CAGCTCCTACTGAACCACCTC	$(AC)_{13}$	186–204	Yakima Yellow	KT992816
	R: AACCAAACAAGCATACATTACTGG				
Cremo34	F: TACACCCGGTCTTCTTCACC	$(AG)_{25}$	142–176	FAM	KT992817
	R: CCGAATCCAGCAATCCTAAG				
Cremo41	F: CCTCGGATGACACCTTCTTC	$(AG)_{13}$	142–166	FAM	KT992818
	R: TAGATGTCATGAGTTGCGGC				
Cremo47	F: CCGACAGCAACCAACGTC	$(AG)_{10}$	152–170	ATTO 550	KT992819
	R: ACTTCACCGGAGGTTACCG				
Cremo54	F: TCAACACTTGCCCTAAACCC	$(ACC)_7$	151–160	ATTO 565	KT992820
	R: CTTTGTTCTGTCTGATGGCG				
Cremo55	F: TTGCATTCTCCATAACTGCG	$(AG)_8$	177–183	Yakima Yellow	KT992821
	R: TGCATTTGAATTTGAAGAAGATG				
Monomorphic loci					
Cremo11	F: TCCCTCTGGTTACACTCATGTC	$(ATC)_5$	149	FAM	KU729207
	R: GAGCTTCATCTGCGATAGGG				
Cremo23	F: CATGACCCTCACCATTAGGAG	$(ATC)_{10}$	154	FAM	KU729208
	R: GCAAACCGGAATGAGACAAC				
Cremo25	F: CATAAAGGGTTGCTTCCAGG	$(ATC)_6$	177	FAM	KU729209
	R: CCAAACCTCCTCGTCTTCTC				
Cremo31	F: ATGGATGACCAATCCTCGTC	$(AAAC)_5$	176	FAM	KU729210
	R: TATCGCGCTCTTGTCACATC				
Cremo32	F: TGTGGAAGGTTCTACTCCCAAG	$(AAG)_5$	110	FAM	KU729211
	R: TCTCTTCAGACTCCGAATCAAC				
Cremo39	F: TGTTGACACTTGAAGAGCGG	$(AAC)_5$	220	FAM	KU729212
	R: TATTGGCACAAACCGCAAC				
Cremo48	F: AAGATCATCACACGCCCAC	$(AG)_{17}$	158	FAM	KU729213
	R: GGAAAGGCACGAGTTCTTTG				
Cremo52	F: TTGTAGGAGGGCCTGAATTG	$(AG)_7$	210	FAM	KU729214
	R: GCCGGAGACCAACATTAAAC				
Cremo58	F: AACAAGATCATCACACGCCC	$(AG)_8$	101	FAM	KU729215
	R: AAATGGTCCCTTGTGGTTTG				
Cremo60	F: AAAGGGACCAAATTGAGCG	$(AT)_6$	138	FAM	KU729216
	R: TCTCGGTGAATCTCTAGCGG				

[a] Optimal annealing temperature for all loci was 58°C.
[b] Fluorescent label marking the forward primer sequence.

were used to generate an indexed paired-end library with pooled equal molar amounts of the genomic DNA of 10 individuals, which was sequenced according to the protocol of the MiSeq Reagent Kit v2 on the MiSeq Desktop Sequencer (Illumina). The sequencing run resulted in 11 million reads, ranging from 100 to 251 bp (average length: 245 bp), which were screened for microsatellite loci.

***Microsatellite screening*—**DNA sequence screening and primer design were conducted with QDD software version 2.1 (Meglecz et al., 2010). In total, 1532 microsatellite loci were identified containing di-, tri-, tetra-, penta-, and hexanucleotide repeat motifs and developed primer combinations had a GC content of 35–60% and a melting temperature (T_m) ranging between 57°C and 60°C. Sixty microsatellite loci were tested for PCR amplification on an initial set of three *C. mollis* DNA samples. Based on visual inspection of agarose gel electrophoresis, 45 of the tested loci showed consistent amplification. To ensure sufficient polymorphism for population genetic analysis, these 45 markers were tested on a set of 14 samples from all five populations. Based on this initial testing, 10 markers proved to be scorable and polymorphic and were assessed further using genomic DNA templates of 50 *C. mollis* specimens from the same populations.

PCRs were performed in 15-µL total volume with the following components: 20 ng of DNA template, 0.16 µM of each forward and reverse primer (Eurofins MWG Operon, Ebersberg, Germany), 1× TaqBuffer S (Peqlab), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, and 0.03 unit Hot *Taq* polymerase (Peqlab). Forward primers were labeled with fluorescent dyes (Table 1), and the reverse primers were extended with seven bases (GTTTCTT) at the 5′ end to reduce stutter bands ("PIG-tailing"; Brownstein et al., 1996). PCR temperature profiles were as follows: 95°C for 1 min; 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and a final extension of 72°C for 7 min. Two loci (Cremo34, Cremo55) were run with the same profile but with a touchdown modification: the annealing temperature started at 60°C and decreased 0.5°C at each of the first 12 cycles, while the last 20 cycles were run with a constant annealing temperature of 54°C. Fragment analysis of the PCR products was carried out on an ABI 3730 sequencer (by Macrogen Europe).

***Microsatellite marker data analysis*—**Individual genotypes were obtained using GeneMarker version 1.95 (SoftGenetics, State College, Pennsylvania, USA) and a GeneScan 500 LIZ Size Standard (Applied Biosystems, Carlsbad, California, USA). As a result, 10 polymorphic loci proved to be useful for population genetic analysis (10 loci were monomorphic, four loci failed to amplify consistently, and 21 loci showed unspecific [stutter] bands) (Table 1). The 10 polymorphic markers provided a total of 82 alleles across 50 samples. Analyzing the genotypes with CERVUS 3.0 (Kalinowski et al., 2007), between two to 17 alleles and a polymorphism information content (PIC)

TABLE 2. Genetic properties of the 10 polymorphic *Crepis mollis* microsatellites from five populations.

	CM01 ($N = 10$)			CM02 ($N = 10$)			CM05 ($N = 10$)			CM06 ($N = 10$)			CM07 ($N = 10$)			Total ($N = 50$)			
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	PIC
Cremo13	2	0.100	0.095	2	0.100	0.095	1	0.000	0.000	2	0.400	0.320	1	0.000	0.000	2	0.120	0.102	0.106
Cremo14	3	0.600	0.645	7	0.556	0.821	4	0.500	0.680	4	0.600	0.565	3	0.222	0.568	7	0.496	0.656	0.768
Cremo15	2	0.100	0.095	2	0.600	0.420	2	0.200	0.180	2	0.400	0.320	2	0.500	0.375	2	0.360	0.278	0.252
Cremo26	8	0.900	0.830	9	0.900	0.865	4	0.400	0.635	7	0.800	0.810	10	0.800	0.880	17	0.760	0.804	0.908
Cremo33	3	0.700	0.505	3	0.600	0.615	3	0.600	0.585	2	0.300	0.375	2	0.300	0.375	4	0.500	0.491	0.479
Cremo34	6	0.800	0.645	9	0.600	0.870	7	0.700	0.730	9	0.300	0.830	4	0.600	0.465	17	0.600	0.708	0.836
Cremo41	7	0.700	0.835	8	0.800	0.820	9	0.600	0.835	10	0.900	0.850	8	0.900	0.830	15	0.780	0.834	0.903
Cremo47	5	0.400	0.660	5	0.800	0.705	5	0.600	0.720	7	0.700	0.805	7	0.700	0.810	10	0.640	0.740	0.800
Cremo54	2	0.200	0.320	1	0.000	0.000	2	0.400	0.420	1	0.000	0.000	2	0.100	0.095	2	0.140	0.167	0.177
Cremo55	4	0.500	0.695	4	0.600	0.655	3	0.200	0.185	5	0.200	0.665	2	0.000	0.480	6	0.300	0.536	0.583

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = sample size; PIC = polymorphism information content.

TABLE 3. Cross-species amplification in three congeneric species with 10 polymorphic microsatellite markers of *Crepis mollis*.

	C. biennis ($n = 6$)				*C. foetida* ($n = 6$)				*C. sancta* ($n = 9$)			
Locus	A	H_o	H_e	Allele size range (bp)	A	H_o	H_e	Allele size range (bp)	A	H_o	H_e	Allele size range (bp)
Cremo13	—	—	—	—	—	—	—	—	—	—	—	—
Cremo14	—	—	—	—	—	—	—	—	—	—	—	—
Cremo15	—	—	—	—	—	—	—	—	—	—	—	—
Cremo26	3	0.500	0.403	160–168	2	0.250	0.219	160–168	—	—	—	—
Cremo33	—	—	—	—	—	—	—	—	—	—	—	—
Cremo34	—	—	—	—	—	—	—	—	—	—	—	—
Cremo41	2	0.000	0.408	124–126	7	0.500	0.833	124–184	5	0.167	0.736	118–206
Cremo47	—	—	—	—	—	—	—	—	—	—	—	—
Cremo54	3	0.714	0.602	157–169	10	0.000	0.278	172–175	1	0.000	0.000	154
Cremo55	—	—	—	—	—	—	—	—	—	—	—	—

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = sample size.

ranging from 0.106 to 0.908 per locus were found (Table 2). GenAlEx 6.5 (Peakall and Smouse, 2006) was used to calculate the observed and expected heterozygosity, which ranged from 0.120 to 0.780 and from 0.102 to 0.834, respectively (Table 2).

Linkage disequilibrium and deviations from Hardy–Weinberg equilibrium (HWE) were tested using GENEPOP (Raymond and Rousset, 1995; Rousset, 2008). Three loci (Cremo14, Cremo47, Cremo55) showed significant deviations from HWE after Bonferroni correction. Larger sample sizes per population are needed to evaluate whether these deviations are due to a Wahlund effect, small population sizes, or null alleles. Tests of linkage disequilibrium revealed that two pairs of loci (Cremo47 and Cremo15, Cremo47 and Cremo33) were significantly linked.

Tests for cross-amplification in the congeneric taxa (*C. biennis*, *C. foetida*, and *C. sancta*) resulted in successful amplification of up to three of the 10 polymorphic loci. For *C. biennis* and *C. foetida*, three loci were amplified and polymorphic. For *C. sancta*, two loci amplified, of which one was monomorphic (Table 3).

CONCLUSIONS

The 10 polymorphic microsatellite markers presented here will be useful to investigate population and conservation genetics of *C. mollis*. This will enable evaluation of inbreeding, neutral genetic differentiation, and gene flow, which are important indices for scientifically informed protective measures of *C. mollis*. Although limited cross-amplification was found, the results suggest the potential of wider applicability of these markers in congeneric species.

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Appendix 1. Voucher specimens, DNA Bank number, and location information of *Crepis mollis* samples and congeneric species used in this study.[a]

Taxon	Population	DNA Bank no.	Collection locality	Geographic coordinates		Collectors and no.	Voucher no.
Crepis mollis (Jacq.) Asch.	CM01	DB 20120–DB 20129	Oelsen, Osterzgebirge, Saxony, Germany	50°47′N	13°56′E	*E. Sossai 12*	B 10 0517356
Crepis mollis	CM02	DB 20140–DB 20149	Hirschsprung, Osterzgebirge, Saxony, Germany	50°47′N	13°44′E	*E. Sossai 13*	B 10 0517357
Crepis mollis	CM05	DB 20159–DB 20168	Johanngeorgenstadt, Erzgebirge, Saxony, Germany	50°25′N	12°43′E	*V. Duwe 14013*	B 10 0517392
Crepis mollis	CM06	DB 20189–DB 20198	Hörnle, Alps, Bavaria, Germany	47°38′N	11°03′E	*V. Duwe 14017*	B 10 0517393
Crepis mollis	CM07	DB 20219–DB 20228	Geigelstein, Alps, Bavaria, Germany	47°42′N	12°21′E	*V. Duwe 14021*	B 10 0517394
Crepis biennis L.	—	DB 60	Reinstädter Grund, Thuringia, Germany	50°48′N	11°32′E	*A. Ueckert, J. Ueckert & C. Oberprieler 9939*	B GT 0000001
Crepis biennis	—	DB 1113	Felbertauern, East Tyrol, Austria	47°7′N	12°29′E	*M. Ristow, B. Gemeinholzer & C. Zidorn 406/07*	B 10 0209389
Crepis biennis	—	DB 1455	Valais Alps, Col de Bretolet, Switzerland	46°8′N	6°47′E	*B. Gemeinholzer 2007*	B 10 0209243
Crepis biennis	—	DB 3770	Odelzhausen, Bavaria, Germany	48°18′N	11°11′E	*T. Dürbye & A. Kirchhoff DÜR 3760*	B 10 0173962
Crepis biennis	—	DB 3961	Nauener Platte, Brandenburg, Germany	52°36′N	12°49′E	*M. Ristow 854/08*	B 10 0340860
Crepis biennis	—	DB 7939	Cornberg, Hesse, Germany	51°2′N	9°51′E	*Kern, Pircher & E. Royl 44*	B GT 0003616
Crepis foetida L.	—	DB 2199	Finsterwalde, Brandenburg, Germany	51°38′N	13°42′E	*M. Ristow 714/08*	B GT 0001444
Crepis foetida	—	DB 3454	Airport, Athens, Greece	37°56′N	23°56′E	*M. Ristow, B. Gemeinholzer, N. Enke, V. Kummer & D. Lauterbach RH-20*	B 10 0326498
Crepis foetida	—	DB 3497	Kattavia, Rhodes, Greece	35°57′N	27°44′E	*N. Enke, A. Suh, H. Phieler & L. Weiß RH-194*	B 10 0326545
Crepis foetida subsp. *foetida*	—	DB 548	Agia Eirini, Cyprus	35°17′N	32°58′E	*R. Hand 5333*	B 10 0209675
Crepis foetida subsp. *communata* (Spreng.) Babc.	—	DB 540	Asinou church, Nikitari, Cyprus	35°4′N	32°59′E	*R. Hand 5257*	B 10 0209682
Crepis foetida subsp. *communata*	—	DB 541	Asinou church, Nikitari, Cyprus	35°4′N	32°59′E	*R. Hand 5263*	B 10 0209666
Crepis sancta (L.) Babc.	—	DB 4934	Lindos, Rhodes, Greece	36°5′N	28°4′E	*M. Ristow RH2-254*	B 10 0341573
Crepis sancta	—	DB 4998	Attavyros, Rhodes, Greece	36°12′N	27°50′E	*M. Ristow, H. Pfestorf & S. Gerull RH2-310*	B 10 0341455
Crepis sancta	—	DB 3452	Airport, Athens, Greece	37°56′N	23°56′E	*M. Ristow, B. Gemeinholzer, N. Enke, V. Kummer & D. Lauterbach RH-17*	B 10 0326501
Crepis sancta	—	DB 3490	Kattavia, Rhodes, Greece	35°57′N	27°45′E	*N. Enke, A. Suh, H. Phieler & L. Weiß RH-151*	B 10 0326554
Crepis sancta	—	DB 3579	Kattavia, Rhodes, Greece	35°57′N	27°46′E	*N. Enke, A. Suh, H. Phieler & L. Weiß RH-174*	B 10 0326561
Crepis sancta	—	DB 3836	Profitis Illias, Rhodes, Greece	36°16′N	27°56′E	*N. Enke RH-276*	B 10 0326171
Crepis sancta	—	DB 3850	Profitis Illias, Rhodes, Greece	36°16′N	27°56′E	*N. Enke RH-293*	B 10 0326217
Crepis sancta	—	DB 5226	Kiotari, Rhodes, Greece	36°3′N	27°58′E	*M. Ristow, S. Hochmuth & J. Schreiber RH-393*	B 10 0326154
Crepis sancta subsp. *bifida* Thell. ex Babc.	—	DB 52	Etschmiadsin town, Armavir province, Armenia	40°9′N	44°19′E	*C. Oberprieler 10032*	B 10 0066672

[a]DNA samples as well as underlying voucher specimens are deposited at the Botanical Garden and Botanical Museum Berlin (B), Germany, and are available via the Global Genome Biodiversity Network (GGBN, 2011).