PRIMER NOTE

DEVELOPMENT OF MICROSATELLITE MARKERS FOR Crepis mollis (ASTERACEAE)¹

VIRGINIA K. DUWE²,⁵, LUDO A. H. MULLER³, THOMAS BORSCH²,³, AND SASCHA A. ISMAIL²,⁴

¹Botanischer Garten und Botanisches Museum Berlin-Dahlem, Dahlem Centre of Plant Sciences, Freie Universität Berlin, Königin Luise-Straße 6–8, 14195 Berlin, Germany; ²Institut für Biologie- Botanik, Dahlem Centre of Plant Sciences, Freie Universität Berlin, Altensteinstraße 6, 14195 Berlin, Germany; and ³School of Biological Sciences, University of Aberdeen, 23 St. Machar Drive, Aberdeen, AB24 3UU Scotland

• 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).

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.) Bab., and their subspecies C. foetida subsp. foetida, C. foetida subsp. communata (Spreng.) Bab., 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

¹Manuscript received 26 February 2016; revision accepted 1 April 2016.

The work was financed by the Federal Agency for Nature Conservation (Bundesamt für Naturschutz [BfN]) as part of the project “Integration of ex situ and in situ measures for the conservation of endangered flowering plants in Germany.” The authors thank H. Fleischer-Notter and L. Botchen for technical assistance. We also thank the Berlin Center for Genomics in Biodiversity Research (BeGenDiv) for performing the Illumina sequencing. This is publication number 023 of BeGenDiv.

²Author for correspondence: v.duwe@bgbm.org
doi:10.3732/apps.1600022
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 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 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).

| Locus | Primer sequences (5’–3’) | Repeat motif | Allele size range (bp) | Fluorescent labelb | GenBank accession no. |
|-------|--------------------------|--------------|------------------------|--------------------|----------------------|
| Polymorphic loci | | | | | |
| Cremo13 | F: GCCCTCATAAGCGCCGACACC | (AGG)$_{6}$ | 246–249 | Yakima Yellow | KT992812 |
| Cremo14 | R: GCCCTCATAAGCGCCGACACC | (AGG)$_{6}$ | 128–155 | FAM | KT992813 |
| Cremo15 | R: TTGGGTTGACATAGCATAGCTGCC | (AAT)$_{7}$ | 116–119 | ATTO 550 | KT992814 |
| Cremo26 | F: AGTGTCTAGCTCTGCTCTG | (AG)$_{8}$ | 180–216 | ATTO 565 | KT992815 |
| Cremo33 | F: CACGGGCTCACCCGACACTCG | (AC)$_{13}$ | 186–204 | Yakima Yellow | KT992816 |
| Cremo34 | F: TACACCAGGCCTTCTCCACCC | (AG)$_{25}$ | 142–176 | FAM | KT992817 |
| Cremo41 | R: CGGAAATCAAGACATAGTAAG | (AT)$_{4}$ | 142–166 | FAM | KT992818 |
| Cremo47 | F: CCGACAGCAACCCAGTC | (AAT)$_{10}$ | 152–170 | ATTO 550 | KT992819 |
| Cremo54 | F: TCAACACTGCTCTAACC | (ACC)$_{7}$ | 151–160 | ATTO 565 | KT992820 |
| Cremo55 | F: TTGATCTCTCATACTCTGG | (AG)$_{8}$ | 177–183 | Yakima Yellow | KT992821 |
| Monomorphic loci | | | | | |
| Cremo11 | F: CCCTCTCCTCTCTCTCTCCG | (ATC)$_{3}$ | 149 | FAM | KU729207 |
| Cremo23 | F: CATGACATCTCCTGCTCAG | (ATC)$_{10}$ | 154 | FAM | KU729208 |
| Cremo25 | F: CAAATTGCTGCTGTGGG | (ATC)$_{8}$ | 177 | FAM | KU729209 |
| Cremo31 | F: AGTGGATAGCAACCTCTTCG | (AAC)$_{3}$ | 176 | FAM | KU729210 |
| Cremo32 | F: TGGGAAAGGTTCTCCTCCCAAG | (AAG)$_{5}$ | 110 | FAM | KU729211 |
| Cremo39 | F: GTTGGTACATCTTGAAGGCGG | (AAC)$_{3}$ | 220 | FAM | KU729212 |
| Cremo48 | F: AGATCATCATACAGCGCCAC | (AG)$_{17}$ | 158 | FAM | KU729213 |
| Cremo52 | F: TGTGAGGAGGCTCTGAATGT | (AG)$_{9}$ | 210 | FAM | KU729214 |
| Cremo58 | F: AAGATCTGATCAATACACCC | (AG)$_{10}$ | 101 | FAM | KU729215 |
| Cremo60 | F: AAAAGGACCAAACTTGGG | (AT)$_{6}$ | 138 | FAM | KU729216 |

*Optimal annealing temperature for all loci was 58°C.

*Fluorescent label marking the forward primer sequence.
### Table 2. Genetic properties of the 10 polymorphic Crepis mollis microsatellites from five populations.

| Locus | CM01 (N = 10) | CM02 (N = 10) | CM05 (N = 10) | CM06 (N = 10) | CM07 (N = 10) | Total (N = 50) |
|-------|---------------|---------------|---------------|---------------|---------------|---------------|
|       | A             | H₀           | Hₑ           | A             | H₀           | Hₑ           | A             | H₀           | Hₑ           | A             | H₀           | Hₑ           | A             | H₀           | Hₑ           | PIC |
| Crem013 | 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 | 0.106 |
| Crem014 | 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 |
| Crem015 | 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 |
| Crem026 | 8 | 0.900 | 0.830 | 9 | 0.900 | 0.865 | 4 | 0.400 | 0.635 | 7 | 0.800 | 0.810 | 10 | 0.800 | 0.830 | 17 | 0.760 | 0.804 |
| Crem033 | 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 |
| Crem034 | 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 |
| Crem041 | 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 |
| Crem047 | 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 |
| Crem054 | 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 | 0.177 |
| Crem055 | 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 |

**Note:** A = number of alleles; Hₑ = expected heterozygosity; H₀ = 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.

| Locus | C. biennis (n = 6) | C. foetida (n = 6) | C. sancta (n = 9) |
|-------|-------------------|-------------------|-------------------|
|       | Allele size range (bp) | Allele size range (bp) | Allele size range (bp) |
|       | A             | H₀           | Hₑ           | A             | H₀           | Hₑ           | A             | H₀           | Hₑ           |
| Crem013 | — | — | — | — | — | — | — | — | — |
| Crem014 | — | — | — | — | — | — | — | — | — |
| Crem015 | — | — | — | — | — | — | — | — | — |
| Crem026 | 3 | 0.500 | 0.403 | 160–168 | 2 | 0.250 | 0.219 | 160–168 |
| Crem033 | — | — | — | — | — | — | — | — | — |
| Crem034 | — | — | — | — | — | — | — | — | — |
| Crem041 | 2 | 0.000 | 0.408 | 124–126 | 7 | 0.500 | 0.833 | 124–184 |
| Crem047 | — | — | — | — | — | — | — | — | — |
| Crem054 | 3 | 0.714 | 0.602 | 157–169 | 10 | 0.000 | 0.278 | 172–175 |
| Crem055 | — | — | — | — | — | — | — | — | — |

**Note:** A = number of alleles; Hₑ = expected heterozygosity; H₀ = 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 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.

LITERATURE CITED

Braithwaite, M. E. 2004. Berwickshire Vice-county Rare Plant Register. Buccleuch Printers Ltd., Hawick, United Kingdom.

Brownstein, M., J. Carpen, and J. Smith. 1996. Modulation of non-templated nucleotide addition by Taq DNA polymerase: Primer modifications that facilitate genotyping. BioTechniques 20: 1004–1010.

Bundesamt für Naturschutz. 2011. FloraWeb: Daten und Informationen zu Wildpflanzen und zur Vegetation Deutschlands. Website http://www.floraweb.de [accessed 5 November 2015].

GGBN. 2011+ (continuously updated). The GGBN Data Portal. GGBN Secretariat, NMNH, Washington D.C., USA. Compiled by GGBN Technical Management, BGBM, Berlin, Germany. Website http://data.ggbn.org [accessed 20 January 2016].

Hegg, G. 1987. DCCXCIX. Crepis L. Pip-pau. In G. Wagenitz [eds.], Illustrierte Flora von Mittel-Europa VI (4,4), ed. 2, 1134–1180. Verlag Paul Parey, Berlin, Germany.

Kalinowski, S. T., M. L. Taper, and T. C. Marshall. 2007. Revising how the computer program Cervus accommodates genotyping error increases success in paternity assignment. Molecular Ecology 16: 1099–1106.

Kilian, N., R. Hand, and E. Von Raar-straube [eds.]. 2009+ (continuously updated). Chichorieae Systematics Portal. Website http://cichorieae.e-taxonomy.net/portal/ [accessed 5 November 2015].

Korneck, D., M. Schnittler, and I. Vollmer. 1996. Rote Liste der Farn- und Blütenpflanzen (Peridophyta et Spermatophyta) Deutschlands. In G. Ludwig and M. Schnittler [eds.], Rote Liste gefährdeter Pflanzen Deutschlands, 21–187. Bundesamt für Naturschutz, Bonn-Bad Godesberg, Germany.

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

Mieuel, H., and E. J. Jager. 1992. Vergleichende Chorologie der Zentraleuropäischen Flora. Gustav Fischer Verlag, Jena, Germany.

O’Reilly, J. 2010. Species account: Crepis mollis. Botanical Society of the British Isles. Website http://ssppaccounts.fsbri.org.uk/content/crepis-mollis-0 [accessed 13 June 2016].

Peakall, R., and P. E. Smouse. 2006. GenAlEx 6: Genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6: 288–295.

Raymond, M., and F. Rousset. 1995. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. Journal of Heredity 86: 248–249.

Rousset, F. 2008. GENEPOP’007: A complete reimplementation of the GENEPOP software for Windows and Linux. Molecular Ecology Resources 8: 103–106.
| Taxon                     | Population | DNA Bank no. | Collection locality | Geographic coordinates | Voucher no.  |
|--------------------------|------------|--------------|---------------------|------------------------|-------------|
| Crepis mollis            | CM06       | DB 20146–DB 20149 | Germany             | 50°47'N 13°44'E         | B 10 03573  |
|                          | CM06       | DB 20154–DB 20158 | Germany             | 47°33'N 12°38'E         | B 10 032646 |
| Crepis sancta            | CM07       | DB 20156–DB 20159 | Germany             | 47°42'N 13°31'E         | B 10 034768 |
| Crepis sancta subsp. bifida | CM07   | DB 20156–DB 20159 | Germany             | 47°42'N 13°31'E         | B 10 034768 |

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