Microsatellite markers for study of the invasive species *Rumex alpinus* (Polygonaceae)

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*Rumex alpinus* (Polygonaceae) is a perennial herb distributed mainly in mountain, subalpine, and alpine zones in Europe. Although the species is considered native in most of Europe, in some areas of Europe it is introduced and considered invasive. These areas include the Orlické Mountains and Krkonoše Mountains in the Czech Republic, the Vosges Mountains in central-western France, and Thuringia and potentially the Black Forest in Germany (Šťastná et al., 2010). It has also been introduced to Great Britain, the United States, and Canada (Šťastná et al., 2010). Populations included in this study were collected in the native range of the species in the French and Austrian Alps. In each population, we collected as many individuals as possible, ensuring that our samples were always at least 2 m apart due to expected clonality of the species. Populations occur in nutrient-rich habitats such as abandoned mountain meadows and along riverbanks (Šťastná et al., 2010). Currently, very little is known about the genetic background of this species. To our knowledge, only one paper has been published to date, using 10 microsatellite markers for phylogenetic reconstruction of five *Rumex* species including *R. alpinus* (Raycheva et al., 2013). These loci were, however, developed for interspecific comparisons and are not suitable for intrapopulation characterization. Despite the invasiveness of this species, no genetic study has been published examining population structure, gene flow between populations, and their relatedness. Although the species is known to be highly clonal (Klimeš, 1992), it is also known to spread both clonally and by seeds (Červenková and Münzbergová, 2009). However, the importance of these two modes of reproduction and their importance for the invasive potential of the species are unknown. We developed and characterized 15 polymorphic markers, which allow for the genetic characterization and further study of this poorly investigated species. Population characterization and estimation of gene flow between populations and an understanding the frequency of these two modes of reproduction will help in designing effective interventions against invasion of this species.

**METHODS AND RESULTS**

**Microsatellite development**

Total genomic DNA of 24 individuals (four individuals per population originating from six populations [Appendix 1]) was extracted from dehydrated leaves using a modified cetyltrimethylammonium bromide (CTAB) method (Lodhi et al., 1994). Concentrations were preserved as described in the protocol, but the total volume of the solutions was reduced 10-fold. The sequencing facility GenoScreen (Lille, France) was used to prepare libraries and design primers. Extracted DNA was pooled for microsatellite library preparation. The fragmented DNA was hybridized with eight probes (TG, TC, AAC, AAG, AGG, ACG, ACAT, and ACTC) to enrich the DNA library. The sequencing was performed by a GS FLX sequencer (454 Life Sciences, a Roche Company, Branford, Connecticut, USA). A total of 55,720 reads were obtained and archived in the GenBank Sequence Read Archive (SRR 5822129). QDD software (Meglécz et al., 2010) with default settings was used to identify microsatellite

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**PREMISE OF THE STUDY:** Polymorphic microsatellite markers were developed to study genetic diversity, population structure, and dispersal strategies of the highly invasive species *Rumex alpinus* (Polygonaceae).

**METHODS AND RESULTS:** Fifteen polymorphic microsatellite loci were developed using a 454 sequencing approach and used to genotype 72 individuals from six populations in Austria and France. All markers were polymorphic in at least one investigated population, and the number of alleles ranged from one to four alleles per locus. Observed and expected heterozygosity ranged from 0.06 to 1.0 and from 0.18 to 0.72, respectively.

**CONCLUSIONS:** These 15 markers will be useful for characterizing dispersal strategies and gene flow assessment between *R. alpinus* populations and other, often weedy *Rumex* species.

**KEY WORDS** invasive species; microsatellites; Polygonaceae; *Rumex alpinus*.
loci and for design of the microsatellite primers. A total of 3089 reads contained microsatellite motifs, and 319 candidate microsatellite loci were identified (Appendix S1) with an average sequence length of 325 bp. Markers belonged to di-, tri-, tetra-, penta-, and hexanucleotide repeats (38.28%, 56.3%, 3.5%, 1.28%, and 0.64%, respectively). Across all candidate loci, 2608 primer pairs (3–15 primer pairs per locus) were designed using the Primer3 algorithm implemented within QDD (Malausa et al., 2011), with amplicon lengths ranging between 90 and 320 bp. For each microsatellite candidate locus, one primer pair was selected for further analysis. Of these, we tested 201 primer pairs (Appendix S1) recommended by GenoScreen to identify polymorphic markers. Primers were synthesized (Sigma-Aldrich, St. Louis, Missouri, USA) with M13 tails preceding the 5′ end of the forward primer sequences (Schuelke, 2000). Four individuals from each of six populations of *R. alpinus* (Appendix 1) were used to test amplification efficiency and polymorphism. DNA amplification was performed in 5-μL reactions consisting of 2.5 μL of QIAGEN Multiplex PCR Master Mix, three types of oligonucleotides (0.125 μL of M13-tagged forward primer, 0.125 μL of species-specific reverse primer, and 0.125 μL of fluorolabeled [5′-FAM] M13 primer [10 μM each in initial volume]), 10 ng of DNA dissolved in 0.5 μL TE buffer, and 1.625 μL of H2O.

The following PCR protocol (Schuelke, 2000) was performed using an Eppendorf Mastercycler Pro S Thermal Cycler (Eppendorf, Hamburg, Germany): an initial denaturation step at 95°C for 15 min; followed by 25 cycles of denaturation (95°C for 15 s), annealing (59°C for 30 s), and extension (72°C for 20 s); followed by 10 cycles of denaturation (95°C for 30 s), annealing (53°C for 45 s), and extension (72°C for 45 s); and a final extension at 72°C for 15 min. During the first 25 cycles specific PCR products are produced, and in the following 10 cycles the fluorescent M13 tag is ligated to the M13 forward primer. A total of 189 primer pairs (94%) were successfully amplified. The majority (74.1%) of loci were monomorphic, and only 49 primer pairs (25.9%) were selected for detailed variability screening on five individuals of *R. alpinus* and five individuals from each of five closely related species (*R. aquaticus*, *R. crispus*, *R. obtusifolius*, *R. sanguineus* L., *R. stenophyllus* Ledeb.). A total of 30 individuals were screened. Based on the variability screening, we chose 15 polymorphic primer pairs for *R. alpinus* species. Most of these were also polymorphic for the closely related species used in the study. GenBank accession numbers for these loci are provided in Table 1.

### Genotyping

Total DNA was extracted from 72 *R. alpinus* individuals from six populations and from 46 individuals from five closely related taxa (Appendix 1) for initial primer screening. Primer sequences, repeat motifs, and PCR product size ranges are given in Table 1. PCR reactions were performed as described above. Each PCR product (1 μL) was mixed with 11 μL of a 120:1 solution of formamide: size standard (GeneScan 500 LIZ; Thermo Fisher Scientific, Waltham, Massachusetts, USA). Fragment lengths were determined by capillary gel electrophoresis with an ABI 3130 Genetic Analyzer using a LabChip 2100 Bioanalyzer (Perkin Elmer, Waltham, Massachusetts, USA). Results were analyzed using GeneMapper Software (Applied Biosystems, Foster City, California). Two control loci were used for each primer set (Ram002 and Ram003) to confirm the absence of stuttering, and 25 cycles were performed for each locus.

### Characteristics of 15 polymorphic loci designed for *Rumex alpinus* genotyping

| Locus | Primer sequences (5′–3′) | Repeat motif | PCR product size range (bp) | GenBank accession no. |
|-------|--------------------------|--------------|-----------------------------|-----------------------|
| Ram32 | F: AGTAAATTCCTTGAACCTCACCACA | (ACCAC)$_{5}$ | 111–156 | MF640423 |
| | R: TGTCGACATGAAGCTGTGAA | | | |
| | R: CGCCATCCGTCAGCAGACT | | | |
| Ram49 | F: GCCAGAAGGGGAGGAGATAC | (GAG)$_{12}$ | 106–112 | MF640424 |
| | R: CCGCGAGATGCTGGACTGTCG | | | |
| Ram92 | F: TGGGATTAATGGAAGGTAGT | (AG)$_{8}$ | 117–127 | MF640425 |
| | R: GCCAGCAGATGAGATGTCG | | | |
| Ram94 | F: GGGAGAGGGTTTGGAGATTC | (GA)$_{17}$ | 117–137 | MF640426 |
| | R: CACAGGCTCACAGGGTCTC | | | |
| Ram103 | F: GTAAAACCGCGCGTAAATGTA | (GA)$_{10}$ | 103–107 | MF640427 |
| | R: GAGATCATGGTGGCCGAGG | | | |
| Ram116 | F: CAAACAAAGGAACGACCGCT | (GA)$_{12}$ | 198–202 | MF640428 |
| | R: TCCTCATTTCTCTCTGATCTC | | | |
| Ram130 | F: ATGGAACCCAGGACCATCA | (AG)$_{13}$ | 184–190 | MF640429 |
| | R: TGAAGTGGCAACATAAAGCCG | | | |
| Ram146 | F: ACCCTAAACCTGGCCCAAT | (CA)$_{13}$ | 302–317 | MF640430 |
| | R: TCACAACGCGTCAAGTCAA | | | |
| Ram165 | F: TGCTACAGGCTGATGCT | (TT)_6 | 149–152 | MF640431 |
| | R: TTTCTCTGCTGGATGATG | | | |
| Ram168 | F: TCGGATCTCTGCCAGAGGA | (AG)$_{9}$ | 107–109 | MF640432 |
| | R: ATTTCCGATTTCCAAATTC | | | |
| Ram173 | F: AAGGACGTTGTTCCAGATAA | (AG)$_{11}$ | 113–115 | MF640433 |
| | R: TCAAGGTTGGTTGGTAGCGTG | | | |
| Ram193 | F: GAGTCTCCCAACCAAGAAGC | (TC)$_{10}$ | 173–189 | MF640434 |
| | R: CTAGTTCTCGCTCTCTACG | | | |
| Ram198 | F: AATGTTGAGTATTGGAGAGGC | (GA)$_{10}$ | 147–169 | MF640435 |
| | R: ACTCTCAAGTGACCGCTC | | | |
| Ram199 | F: CCGAGCTATTTCCAGATAC | (CA)$_{18}$ | 165–177 | MF640436 |
| | R: TTCTTGACCGTGAATTGCCTG | | | |
| Ram200 | F: AACTGCGCCACATGGAGGT | (AG)$_{14}$ | 238–248 | MF640437 |
| | R: GTGCGACACTGAGAAACCCTG | | | |

*Optimal annealing temperature was 59°C for all loci.

M13 tag (Schuelke, 2000) addition to 5′ terminator.
GeneMapper 4.0 (Thermo Fisher Scientific). Using GenAlEx 6.5 (Peakall and Smouse, 2012), we calculated observed heterozygosity, expected heterozygosity, and number of alleles. Number of alleles ranged from one to four and observed and expected heterozygosities ranged from 0.06 to 1.0 and from 0.18 to 0.72, respectively. GENEPOP 4.2 (Rousset, 2008) was used to calculate deviations from Hardy–Weinberg equilibrium and linkage disequilibrium. Significant linkage disequilibrium was found within four populations following Bonferroni correction (Crozet population: loci 94/198 and 165/173; Katschberg population: loci 198/94, 193/94, 198/32, and 94/32; Heiligenblut population: loci 199/32, 193/32, and 198/94; Ferleiten population: 198/94 and 200/92). Significant deviation from Hardy–Weinberg equilibrium was found mainly in the Crozet and Katschberg populations (Table 2), which indicates strong clonality. This indication is also supported for the Crozet population by lower observed heterozygosity than expected for each locus of this population (Table 2).

We also tested cross-amplification of these loci in five other Rumex taxa: R. aquaticus, R. crispus, R. obtusifolius, R. sanguineus, and R. stenophyllus. Tests for cross-amplification and polymorphism in the five congeneric taxa resulted in successful genotyping of up to 14 of the 15 loci (Table 3). These results demonstrate that these primer pairs may be of broad utility throughout the genus Rumex.

CONCLUSIONS

We developed and successfully amplified 15 polymorphic markers in several Rumex taxa, including R. alpinus, R. aquaticus, R. crispus, R. obtusifolius, R. sanguineus, and R. stenophyllus. These polymorphic loci will be valuable for the future study of R. alpinus population genetic composition and its influence on the invasiveness of the species.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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APPENDIX 1. Accession information for *Rumex* species in this study.

| Species       | Collection locality/Population code | Country         | n   | Latitude | Longitude |
|---------------|-----------------------------------|-----------------|-----|----------|-----------|
| *R. alpinus* L. | Crozet               | France          | 16  | 46.2866339 | 6.0107875 |
| *R. alpinus*   | Hinterstoden-Hoss      | Austria         | 5   | 47.696796  | 14.153384 |
| *R. alpinus*   | Spital am Pyhrn        | Austria         | 5   | 47.638855  | 14.354099 |
| *R. alpinus*   | Katschberg             | Austria         | 15  | 47.055959  | 13.616254 |
| *R. alpinus*   | Heiligenblut           | Austria         | 15  | 47.064204  | 12.827788 |
| *R. alpinus*   | Ferleiten              | Austria         | 16  | 47.134005  | 12.807474 |
| *R. aquaticus* L. | Markvartice         | Czech Republic  | 10  | 50.4294492 | 15.1982517|
| *R. crispus* L. | Markvartice           | Czech Republic  | 10  | 50.4294492 | 15.1982517|
| *R. obtusifolius* L. | Markvartice      | Czech Republic  | 10  | 50.4294492 | 15.1982517|
| *R. sanguineus* L. | Markvartice       | Czech Republic  | 6   | 50.4294492 | 15.1982517|
| *R. stenophyllus* Ledeb. | Cemonicice      | Czech Republic  | 10  | 49.91623   | 14.30901  |

Note: n = number of individuals.

*Leaf samples of 5–16 individuals per population were collected in the field and dried (silica gel) before performing DNA extraction. Herbarium vouchers, leaf samples, and DNA extracts are deposited at the Institute of Botany of the Czech Academy of Sciences (PRA), Příhonice, Czech Republic.*

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