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PRIMER NOTE

Development and characterization of novel SSR markers in the endangered endemic species *Ferula sadleriana*

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PREMISE: *Ferula sadleriana* (Apiaceae) is a polycarpic, perennial herb with a very limited range and small populations. It is listed as "endangered" on the IUCN Red List of Threatened Species. Microsatellite markers can contribute to conservation efforts by allowing the study of the genetic structure of its shrinking populations.

METHODS AND RESULTS: We used a microsatellite-enriched library combined with an Illumina sequencing approach to develop simple sequence repeat markers in our target species. Out of 44 tested primer pairs, 22 provided specific products, and 13 showed heterologous amplification in the target species. Cross-species amplification was achieved at 20 and 19 loci in two congeneric species, *F. soongarica* and *F. tatarica*, respectively.

CONCLUSIONS: The primers described here are the first tools that enable the population genetic characterization of *F. sadleriana*. Our results suggest a wider applicability in the genus *Ferula*.

KEY WORDS Apiaceae; conservation genetics; endemic; *Ferula sadleriana*; microsatellite; Umbelliferae.

Ferula L. is a genus of the Apiaceae family containing approximately 170 species with a geographic range extending from northern Africa to Central Asia (Pimenov and Leonov, 1993). It includes many species used in traditional medicine and numerous species that are endemic, especially in the Central Asian region (Kurzyna-Młynik et al., 2008). *Ferula sadleriana* Ledeb. is an iteroparous perennial herb with a range confined to the Carpathian Basin; it is assumed to be an interglacial relict with special biogeographical importance in the region (Lendvay and Kalapos, 2014). Despite its uniqueness, nothing is known about its phylogenetic placement as it has not been included in the latest comprehensive phylogeny of the genus (Panahi et al., 2018). The species is restricted to a mosaic habitat of rocky, dry grasslands, steppe slopes, and Pannonian karst white oak low woods (Kalapos and Lendvay, 2009). Only eight populations are known, centered in the hilly regions of northern Hungary and southern Slovakia with a satellite occurrence in Transylvania (central Romania). Population sizes range from less than 50 to 5000 individuals (Kalapos and Lendvay, 2009). Due to the small population sizes and the restricted distribution of the species, it is strictly protected in all three countries of its occurrence (Lendvay and Kalapos, 2014). It is classified as "endangered" in the IUCN Red List of Threatened Species (Király et al., 2011) and is included in the Annex II and IV of the Habitats Directive of the European Union (Council of the European Communities, 1992).

To facilitate the conservation efforts focusing on this unique species, we developed 13 polymorphic simple sequence repeat (SSR) markers that enable the study of the conservation genetics of *F. sadleriana*. We tested cross-amplification efficacy in two species (*F. soongarica* Pall. ex Schult. and *F. tatarica* Fisch. ex Spreng.) of the Eurasian steppe zone that form a monophyletic group with our target species within the genus (G. Sramkó, unpublished data). We are convinced that the markers described here will be useful in population genetic studies of this large genus.

METHODS AND RESULTS

Genomic DNA was extracted from populations of *F. sadleriana* and putative relatives (Appendix 1) following a modified cetyltrimethylammonium bromide (CTAB) protocol (Sramkó et al., 2014) using leaf material dried in silica gel. The Nextera Library Preparation Kit (Illumina, San Diego, California, USA) was used to construct the library from the equimolar mix of DNA from three individuals (Appendix 1) following the manufacturer's protocol. Paired-end reads 250 bp in length were obtained using an Illumina MiSeq system with MiSeq Reagent Kit version 2. Sequencing reads were analyzed by QDD version 3.1.2 (Meglécz

et al., 2014) using default settings to detect microsatellites and design primers. From the 9111 sequences with successful primer design, the following criteria were used to select primer pairs for laboratory testing: (i) the sequence contained only pure microsatellites in the target region with at least eight repeats; (ii) did not contain repeats of $(AT)_n$; (iii) the primer alignment score to the amplified sequence was lower than 6; (iv) primers were at least 10 bases away from the microsatellite motif; (v) consensus sequences are based at most on three reads; (vi) no transposable elements are detected by RepeatMasker version 1.317 (Smit et al., 2013–2015); and (vii) no BLAST hit to non-Viridiplantae sequences in GenBank.

In total, 222 potential loci were identified, out of which the first 44 were selected for initial screening. Specific PCR amplification of the 44 loci was tested on a DNA sample of one individual from the Turda Gorge population (Appendix 1), implementing a temperature gradient PCR protocol. The success of PCR was evaluated on a chilled 2% agarose gel. The PCR mixture contained 1× DreamTaq Green Buffer, 0.2 mM dNTP (each), 1 mg/mL bovine serum albumin, 0.5 μM of each primer, 0.05 units DreamTaq Green DNA Polymerase, and 2 ng of template DNA for a final volume of 10 μL (all PCR reagents were purchased from Thermo Scientific, Carlsbad, California, USA). The cycling regime of the PCR protocol was: 94°C for 3 min; 40 cycles of 15 s at 94°C, 30 s

TABLE 1. Characteristics of 22 microsatellite loci developed in *Ferula sadleriana*.

Locus[a]	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	Fluorescent dye	GenBank accession no.
FSad01[d]	F: TGCGATGTTGAAGATAAACGGC	$(AG)_{13}$	240	—	MN603946
	R: ATCGGCACCACTCACAGTAG				
FSad03[d]	F: ATCAACATCTATTATCAGTCATCCTTC	$(GA)_{17}$	140	—	MN603947
	R: AAATGACCCTGATCGTTGAGG				
FSad04[d]	F: GTGAGCACTGGATACCGGAC	$(TG)_9$	94	—	MN603948
	R: TCTGCTACCAACAGTCCTGG				
FSad06[b]	F: CCCGCATTACATTATTTGTAGTCG	$(GA)_9$	111–121	VIC	MK393172
	R: CGTCCTCGTCACCAGATAAGC				
FSad07[b]	F: AGGAGGTGTTGTTGACCCAG	$(GT)_{10}$	216–226	VIC	MK393173
	R: CCACTTCACAAATCTAAATTCCTACAC				
FSad09[c]	F: CTGTCGCCGGTGGTGATG	$(AG)_9$	193–249	6-FAM	MK393174
	R: CCACTGATCACTACAGCGCC				
FSad10[d]	F: GGAAGGATAAGAAATATCAATCGGACG	$(TG)_{13}$	151	—	MN603949
	R: TGCTTTATTGCGGAGAAAGTTCC				
FSad11[c]	F: TGGTTCTCATTCGAGCACATG	$(AG)_{11}$	156–168	VIC	MK393175
	R: CTTCCTCCACTGACCGTGAG				
FSad12[b]	F: TGCCATGCATACTGTGTAACAC	$(CT)_9$	288–294	6-FAM	MK393176
	R: GCTCTTGATTCTCATCTGAAACAC				
FSad13[d]	F: CATCTGAGCGAGGCCGAC	$(GA)_{10}$	192	—	MN603950
	R: TATCTCCTCTTCTTCGCCACC				
FSad20[d]	F: GCAATGGCTTCAATCGGTTC	$(TC)_9$	168	—	MN557388
	R: ACCGCAATAGAAGCTCTAAGAGG				
FSad25[b]	F: AACGATGTCGCACTTCGGAC	$(CT)_{15}$	171–211	NED	MK393177
	R: CAACGGGAACAAATCATCAGCC				
FSad26[d]	F: ACCAGGTCCCACTCCCTTTC	$(TC)_9$	241	—	MN557389
	R: AGAATTGTGAACATCAAAGACCC				
FSad28[d]	F: ATTTACCGGCGGTATGACCC	$(CT)_{10}$	135	—	MN603951
	R: TGGTCGAAGCATGGTTGGAG				
FSad32[d]	F: AGACTATCCAGAGGACCGGC	$(GAA)_9$	240	—	MN603952
	R: TTGTTCTGCCAGAGATCCCG				
FSad33[c]	F: TTTCTACCATCGCCATCTACCAC	$(CT)_{10}$	282–292	PET	MK393178
	R: AGAGTAAGTATCTCCAGATGTTCTTG				
FSad36[c]	F: AGTTGCCAAGATTTGTGTCTAGC	$(CA)_{10}$	135–143	NED	MK393179
	R: TGTGCCTTTGTACTGTAGTGG				
FSad37[b]	F: GACATAAGTTGGTTGAGTTAGTTAGG	$(CT)_{11}$	115–159	6-FAM	MK393180
	R: ACACTCTATTTGATGGAGTCACC				
FSad39[c]	F: ACACATAACCCAACGACTACGG	$(TC)_{10}$	228–240	NED	MK393181
	R: TGTTACCGTCTTCTCCGACC				
FSad40[c]	F: AGCGCAGGAATTGAATTGGC	$(AAG)_{10}$	129–147	6-FAM	MK393182
	R: TTCCCACCCATAGACTGCTG				
FSad42[b]	F: TCAGTCATCATCCTTTCCTTCTC	$(TC)_9$	134–160	PET	MK393183
	R: GACATGCTATGACCATCTGAATTC				
FSad43[c]	F: TGGCAAAGAGTCAGCAATGC	$(TAGA)_9$	182–234	PET	MK393184
	R: ACTAGGTGTACATGAAGACACGG				

[a]Annealing temperature was 64°C for all primers.
[b]Included in Multiplex 1.
[c]Included in Multiplex 2.
[d]Monomorphic in our preliminary test and therefore omitted from further analyses.

at 56°C–64°C, and 30 s at 72°C; with a final extension at 72°C for 14 min. After this step, 22 primer pairs (Table 1) remained that presented specific products at the same annealing temperature of 64°C. These loci were further tested for polymorphism at the population level on five individuals from the Pilis Hill population (Appendix 1). The same PCR mixture and evaluation method was used as described above. Cycling regime was also the same except setting annealing temperature to 64°C. Thirteen loci proved to be polymorphic at the population level based on visual inspection of the agarose gel. These loci were selected for further analysis, and their forward primers were labeled fluorescently at their 5′ end (Table 1). All 13 selected loci were PCR amplified using the PCR conditions described above.

Three populations (n = 16, 20, and 18) were used to test the applicability of these loci for population genetics (Table 2). The fluorescently labeled PCR products were analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Multiplexing was carried out equimolarly including six loci in each mix, according to their fragment length and label type (Table 1). One microliter of the multiplexed PCR products was added to 0.25 μL of GeneScan 500 LIZ Size Standard (Applied Biosystems) and 14.75 μL Hi-Di formamide (Genetic Analysis Grade, Applied Biosystems) before analysis. PeakScanner version 1.0 (Applied Biosystems) was used to carry out genotype calling manually.

Here we report numbers of alleles and private alleles, and levels of expected and observed heterozygosity. Genotypes were analyzed with the software GenAlEx version 6.5 (Peakall and Smouse, 2012). Deviations from Hardy–Weinberg equilibrium (HWE) and measures of linkage disequilibrium among loci were calculated using GENEPOP version 4.2 (Raymond and Rousset, 1995; Rousset, 2008). Null alleles were assessed by FreeNA (Chapuis and Estoup, 2007). The Markov chain Monte Carlo parameters were left at default settings when used for the probability test of HWE. Allele numbers ranged from 3–14 across all populations, with an average number of private alleles of 1.4 (Turda Gorge population), 0.1 (Pilis Hill population), and 0.9 (Bélkő population). Expected heterozygosity values ranged from 0.095 to 0.869, observed heterozygosity values ranged between 0.05 and 0.938, and deviations from HWE were present in all three populations, most prevalent at loci FSad37 and FSad33 (Table 2). This pattern is likely the result of the presence of null alleles (Table 2) and requires adjustment of allele frequencies (Chapuis and Estoup, 2007). Linkage disequilibrium was observed between loci FSad12 and FSad33 (P = 0.001), FSad25 and FSad40 (P = 0.001), and FSad40 and FSad42 (P = 0.004) across all populations.

Cross-species amplification was tested in three populations of *F. soongarica* (n = 6) and three populations of *F. tatarica* (n = 4), both of which are close relatives of *F. sadleriana*. Using the same PCR conditions and a chilled 2% agarose gel, 20 and 19 of the 22 original loci amplified and presented a specific product, with apparent polymorphism in most loci, in *F. soongarica* and *F. tatarica*, respectively (Table 3).

TABLE 3. Cross-amplification success (showing allele size range in base pairs) of 22 microsatellite loci developed in *Ferula sadleriana* in closely related *Ferula* species.[a]

Locus	*Ferula soongarica* (n = 6)	*Ferula tatarica* (n = 4)
FSad01	256–290	228–284
FSad03	130–132	138–140
FSad04	113–116	117
FSad06	139–154	137–151
FSad07	212–254	212–233
FSad09	201–230	202–255
FSad10	180	179–186
FSad11	169–186	173–189
FSad12	300–319	286–337
FSad13	206–211	203–207
FSad20	172–179	—
FSad25	200–243	194–221
FSad26	217–238	216–227
FSad28	—	—
FSad32	—	—
FSad33	279–310	290–322
FSad36	137–145	138–140
FSad37	137–165	141–177
FSad39	221–251	231–249
FSad40	199–206	201–205
FSad42	190–203	193–228
FSad43	157	192–217

Note: — = unsuccessful amplification; n = number of individuals.
[a]Locality and voucher information are provided in Appendix 1.

TABLE 2. Genetic properties of the 13 polymorphic SSR markers developed in *Ferula sadleriana*.[a]

	Total (n = 54)			Turda Gorge (n = 16)					Pilis Hill (n = 20)					Bélkő (n = 18)				
Locus	A	H_o	H_t	A	A_p	Null	H_o	H_e	A	A_p	Null	H_o	H_e	A	A_p	Null	H_o	H_e
FSad06	6	0.519	0.786	5	0	0.083	0.500	0.621	5	0	0.193	0.400*	0.739	6	1	0.072	0.667	0.792
FSad37	10	0.283	0.808	7	4	0.352	0.133*	0.742	5	0	0.277	0.250*	0.738	5	1	0.176	0.444*	0.736
FSad42	5	0.407	0.503	4	1	0.000	0.563	0.584	2	0	0.000	0.100	0.095	4	1	0.011	0.611	0.631
FSad25	14	0.704	0.840	8	3	0.057	0.688	0.822	4	0	0.000	0.650	0.558	11	4	0.019	0.778	0.840
FSad07	5	0.444	0.732	5	1	0.000	0.750*	0.699	4	0	0.243	0.300*	0.716	4	0	0.119	0.333	0.539
FSad12	4	0.426	0.547	3	1	0.087	0.375	0.508	2	0	0.166	0.250	0.499	3	1	0.000	0.667	0.586
FSad36	3	0.352	0.339	3	1	0.000	0.250	0.225	2	0	0.000	0.400	0.375	2	0	0.000	0.389	0.375
FSad40	7	0.556	0.748	6	1	0.002	0.625	0.699	4	0	0.066	0.400	0.546	6	1	0.020	0.667	0.653
FSad11	7	0.593	0.752	7	1	0.006	0.813	0.797	5	0	0.000	0.600	0.611	3	0	0.081	0.389	0.468
FSad43	8	0.500	0.777	6	2	0.270	0.286*	0.763	5	0	0.080	0.550	0.700	5	1	0.000	0.611	0.583
FSad09	14	0.667	0.881	9	3	0.097	0.625*	0.813	7	1	0.035	0.800	0.823	10	2	0.170	0.556*	0.869
FSad39	6	0.704	0.737	6	0	0.000	0.938	0.779	4	0	0.000	0.700*	0.648	6	0	0.111	0.500*	0.698
FSad33	3	0.130	0.626	3	0	0.218	0.188*	0.529	2	0	0.300	0.050*	0.499	3	0	0.263	0.167*	0.586

Note: A = number of alleles; A_p = number of private alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; H_t = expected heterozygosity among all populations; n = number of individuals.
[a]Locality and voucher information are provided in Appendix 1.
*Significant deviation from Hardy–Weinberg equilibrium at P < 0.05 level.

CONCLUSIONS

We developed 22 SSR markers in *F. sadleriana*, out of which 13 proved to be polymorphic in the studied populations. As the first genetic markers developed for this endangered species, they represent key tools in the population genetics and therefore conservation biology of these plants. Most markers provided specific products in two congeneric species, suggesting their wider applicability in the genus, which is currently completely lacking such markers.

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DATA AVAILABILITY

Raw sequencing reads were deposited in the European Nucleotide Archive (ENA) under the study accession number PRJEB35561 ("*Ferula sadleriana* microsatellite discovery"). Sequence information for the primers described here has been deposited in the National Center for Biotechnology Information's GenBank database, and accession numbers are provided in Table 1.

LITERATURE CITED

Chapuis, M.-P., and A. Estoup. 2007. Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution* 24: 621–631.

Council of the European Communities. 1992. Council Directive 92/43/ EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora. Website http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:1992L0043:20070101:EN:PDF [accessed 25 September 2019].

Kalapos, T., and B. Lendvay. 2009. A magyarföldi husáng (*Ferula sadleriana*) populációinak állapotfelmérése 2008-ban [Survey of *Ferula sadleriana* populations in 2008]. *Természetvédelmi Közlemények* 15: 486–492.

Király, G., V. Ferakova, P. Mereďa, I. Hodálová, and P. Eliáš. 2011. *Ferula sadleriana*. The IUCN Red List of Threatened Species: e.T161991A5524668. Website https://doi.org/10.2305/IUCN.UK.2011-1.RLTS.T161991A5524668.en [accessed 25 September 2019].

Kurzyna-Młynik, R., A. A. Oskolski, S. R. Downie, R. Kopacz, A. Wojewódzka, and K. Spalik. 2008. Phylogenetic position of the genus *Ferula* (Apiaceae) and its placement in tribe Scandiceae as inferred from nrDNA ITS sequence variation. *Plant Systematics and Evolution* 274: 47–66.

Lendvay, B., and T. Kalapos. 2014. Population dynamics of the climate-sensitive endangered perennial *Ferula sadleriana* Ledeb. (Apiaceae). *Plant Species Biology* 29: 138–151.

Meglécz, E., N. Pech, A. Gilles, V. Dubut, P. Hingamp, A. Trilles, R. Grenier, and J. F. Martin. 2014. QDD version 3.1: A user-friendly computer program for microsatellite selection and primer design revisited: Experimental validation of variables determining genotyping success rate. *Molecular Ecology Resources* 14: 1302–1313.

Panahi, M., Ł. Banasiak, M. Piwczyński, R. Puchałka, M. Reza Kanani, A. A. Oskolski, D. Modnicki, et al. 2018. Taxonomy of the traditional medicinal plant genus *Ferula* (Apiaceae) is confounded by incongruence between nuclear rDNA and plastid DNA. *Botanical Journal of the Linnean Society* 188: 173–189.

Peakall, R., and P. E. Smouse. 2012. GenALEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research–an update. *Bioinformatics* 28: 2537–2539.

Pimenov, M., and M. Leonov. 1993. The genera of the Umbelliferae: A nomenclator. Website https://www.cabdirect.org/cabdirect/abstract/19951608740 [accessed 20 December 2018].

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 re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* 8: 103–106.

Smit, A. F. A., R. Hubley, and P. Green. 2013–2015. RepeatMasker Open-4.0. Website http://www.repeatmasker.org [accessed 1 September 2014].

Sramkó, G., A. Molnár V., J. A. Hawkins, and R. M. Bateman. 2014. Molecular phylogeny and evolutionary history of the Eurasiatic orchid genus *Himantoglossum* s.l. (Orchidaceae). *Annals of Botany* 114: 1609–1626.

APPENDIX 1. Geographic and voucher information of *Ferula* populations represented in this study.

Species	*N*	Location, ISO country code	Geographic coordinates	Voucher[a]
Ferula sadleriana	20	Pilis Hill, HU[b,d]	47°40′51.17″N, 18°52′47.28″E	DE-Soo-45690
Ferula sadleriana	18	Pisznice, HU[b]	47°41′59.06″N, 18°29′27.49″E	DE-Soo-45689
Ferula sadleriana	10	Konyári plateau, SK[b]	48°34′29.35″N, 20°23′09.96″E	DE-Soo-45687
Ferula sadleriana	16	Turda Gorge, RO[c]	46°33′31.27″N, 23°40′56.32″E	DE-Soo-45691
Ferula sadleriana	18	Bélkő Hill, HU	48°02′33.40″N, 20°22′32.20″E	DE-Soo-48196
Ferula soongarica	3	Katon-Karagay, KZ	49°13′14.66″N, 85°46′55.45″E	DE-Soo-45033
Ferula soongarica	2	Markakol: Uspenka, KZ	48°30′11.92″N, 85°53′16.15″E	DE-Soo-45692
Ferula soongarica	1	Ust'-Kamenogorsk, KZ	50°02′51.72″N, 81°23′19.39″E	DE-Soo-45028
Ferula tatarica	2	Strilcovskaya steppe, UA	49°17′03.87″N, 40°02′04.73″E	DE-Soo-45688
Ferula tatarica	1	Kalach-na-Donu, RS	48°41′16.44″N, 43°27′13.03″E	DE-Soo-45038
Ferula tatarica	1	Danilovka valley, RS	50°34′24.40″N, 45°39′36.05″E	DE-Soo-45037

Note: ISO = International Organization for Standardization; *N* = number of individuals sampled.
[a]Voucher specimens are deposited at the herbarium of the University of Debrecen (DE), Debrecen, Hungary.
[b]One individual from this population was used to construct the initial Nextera primer development library.
[c]Specific amplification of all 44 primers designed was tested on one individual of this population.
[d]Population-level variability of specific primers was screened on five individuals of this population.

