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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF CHLOROPLAST MICROSATELLITE MARKERS IN A FINE-LEAVED FESCUE, *FESTUCA RUBRA* (POACEAE)[1]

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- *Premise of the study:* Chloroplast microsatellite markers were developed for *Festuca rubra* to examine its population genetic characteristics, taxonomy, and coevolution with its endophyte *Epichloë festucae.*
- *Methods and Results:* Thirteen polymorphic markers were identified from the chloroplast genome of a *F. ovina* accession and intergenic chloroplast sequences of *F. rubra* accessions. They amplified a total of 65 alleles in a sample of 93 individuals of *F. rubra* originating from six different populations located in the Faroe Islands, Finland, Greenland, Norway, and Spain.
- *Conclusions:* The developed microsatellite primer pairs can be used by researchers in population genetic and taxonomic studies, and by plant breeders in breeding programs on grasses.

Key words: agriculture; breeding; *Epichloë festucae*; *Festuca rubra*; pasture grass; Poaceae; population genetics; taxonomy.

Festuca rubra L. (red fescue) belongs to fine-leaved *Festuca* sect. *Aulaxyper* s.l. clade (*F. rubra* group, family Poaceae). *Festuca rubra* is perennial, rhizomatous, and highly interfertile with multiple ploidy levels (Dirihan et al., 2013). It is one of the agriculturally most important turfgrasses, widely cultivated in temperate regions (Gould and Shaw, 1983), with a number of commercial cultivars. The genus *Festuca* L. contains by recent estimates from 450 to more than 500 species with nearly global distribution (Lu et al., 2006; Darbyshire and Pavlick, 2007). The taxonomy of the genus *Festuca* is problematic and contentious (Darbyshire and Pavlick, 2007). The International Plant Names Index (IPNI) catalogues worldwide 37 subspecies, 36 varieties, and 19 forms of *F. rubra*. It has encountered a wide and rapid inter- and postglacial expansion around the world, and occupies a diverse range of ecological conditions (Inda et al., 2008). Consequently, *F. rubra* is morphologically highly variable, and plants falling into morphologically distinguishable categories are often inconsistently classified as both species and subspecies. The success of *F. rubra* is often linked with the systemic and vertically transmitted endophyte, *Epichloë festucae* Leuchtm., Schardl & Siegel, because the endophyte infection has been demonstrated to provide a selective advantage or disadvantage to the host plant depending on prevailing selection pressures (Wäli et al., 2009; Saikkonen et al., 2010).

Chloroplast microsatellites or simple sequence repeats (cpSSR) are used as effective tools in evolutionary, population genetic, and phylogeographic studies (Provan et al., 2001; Ebert and Peakall, 2009). In grasses, chloroplast microsatellite markers have been previously developed for *Lolium perenne* L. and tested also in fine-leaved *F. rubra*, resulting in five amplifiable polymorphic markers in *F. rubra* with low levels of intraspecific variation (McGrath et al., 2006). In fine-leaved fescues, cross-amplification problems of the chloroplast markers designed for other grass taxa can be caused by the smaller plastid genome size of fine-leaved *Festuca* due to a larger number of deletions within the intergenic regions compared to other grasses (Hand et al., 2013). However, no species-specific cpSSR markers have been developed for fine-leaved *Festuca* so far. In our study, we needed a greater number of polymorphic chloroplast markers to address our study aims, such as population genetic characteristics and the coevolution patterns of *F. rubra* with its endophyte *E. festucae*.

METHODS AND RESULTS

Chloroplast DNA is conserved, nonrecombinant, uniparentally inherited, and effectively haploid, and it generally lacks heteroplasmy, thus being ideal for marker development, as the flanking regions are typically conserved (Provan et al., 2001; Hand et al., 2013). In our study, the starting point of the cpSSR marker development for *F. rubra* was the plastome sequence of taxonomically

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TABLE 1. Characteristics of 14 intergenic chloroplast microsatellite markers developed for the grass *Festuca rubra*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)[b]	Position	GenBank accession no.
FR15cpSSR	F: CCATCTCTCCCCGTTCCAAA R: TTGTCTCTCGGCCAATATTGA	$(T)_{11}C(T)_3,(T)_6$	203–212	trnS-GCU/psbD	JX871940
FR16cpSSR	F: AGCGCACTATTGTAAATCGAAGT R: AGTTTGCCAGGGGTACAACT	$(TAT)T(TAT)_4,(T)_8$	229–234	trnS-GCU/psbD	JX871940
FR17cpSSR	F: GCCGCATCAATCGAGGATAC R: TCCGACAACCTCAGGAGAAA	$(A)_8C(A)_{13}$	217–222	ycf3/trnS-GGA	JX871940
FR19cpSSR	F: TAAGCAAGCGGTGTCTCTCA R: ACAATCAAGTCCGTAGCGTC	$(A)_{12}$	174–180	trnT-UGU/trnL-UAA	JX871940
FR20cpSSR	F: TCCTCGTGTCACCAGTTCAA R: AGCCTAATCTCACCTCCTTCTG	$(A)_7(TA)_3,(A)_7$	245–257	trnF-GAA/ndhJ	JX871940
FR21cpSSR	F: AGGACTAATCTCTGCAGTATAATGAGA R: TCCATCTTGCGAATTACTACCTTG	$(A)_9G(A)_9,(GA)_4,(T)_7$	246–260	ndhC/trnV-UAC	JX871940
FR23cpSSR	F: TCCACTTTCTTTTACGCTTCTGT R: AGCAGCCAGTAGAAAACCGA	$(A)_{13},(T)_7$	182	psbE/petL	JX871940
FR24cpSSR	F: CCGTCTTATATAGGGGATAGGCT R: TGCCGCAAATAAATCCTTCTTTC	$(AT)_5,(AT)_6,(AT)_3,(AT)_3,(AT)_3,(A)_7$	292–301	ndhF/rpl32	JX871940
FR26cpSSR	F: AGTCCCCTTAGTGGTCCCTA R: TCCGTAACCGTGCATGAATT	$(T)_{12},(T)_6$	186–190	atp1/atpH	JX871940
FR27cpSSR	F: GGAGGAATTGCGGGTTTTCT R: TACCTCGCCTGAACCTAAGC	$(T)_7C(T)_6,(TTC)_4$	198–201	petA/psbJ	JX871940
FR28cpSSR	F: AGGAGAACACAGAGTCATAGCA R: CTCTCCCCGCCCTACTTTAT	$(A)_{11}$	122–124	trnT/trnL	EF585096
FR29cpSSR	F: TCAATTTGATATGGCTCAGAGGA R: TGCTATGACTCTGTGTTCTCCT	$(AT)A(AT)_5$	191–200	trnT/trnL	DQ336857.1
FR30cpSSR	F: CAGCAATAGTGTCCTTGCCC R: GATTGCCGAGGAATTGAGAGA	$(T)_4C(T)_9CA(T)_4$	226–231	rps8/rpl14	HM173006
FR31cpSSR	F: TGACAAAGGAGTGCGAAGAG R: CTTGTGCATCATCCTAGTAGAGT	$(C)_9$	250–254	trnL/trnF	EF593001

[a]Annealing temperature = 56°C.

[b]Size ranges are based on 93 samples representing European populations located in Finland, the Faroe Islands, Greenland, Norway, and Spain (n = 12–18 for each population); see Appendix 1 for population information.

closely related *F. ovina* L. (Inda et al., 2008), which is the only available complete fine-leaved *Festuca* plastome sequence (Hand et al., 2013). The chloroplast genome sequence of *F. ovina* (GenBank accession no. JX871940, length 133,165 bp) was downloaded from GenBank and searched for ≥7 mononucleotide repeats and ≥3 di-, tri-, and tetranucleotide repeats using MSATFINDER version 2.0.9 (Thurston and Field, 2005). A total of 569 repeat motifs were identified in the chloroplast genome, among which the most frequent types were mononucleotide (44%) and dinucleotide (46%) repeats, while tri- (8%) and tetranucleotide (1%) repeats were rare. In addition, the GenBank accessions of *F. rubra* intergenic chloroplast sequences were downloaded and similarly searched for mono-, di-, and trinucleotide repeats. The sequences containing repetitive motifs were aligned among accessions to reveal variation among individuals. The selection of the regions with repetitive motifs for primer design was based on the repeat length being as long as possible, the region located within an intergenic region and being a known mutational hot spot region (Hand et al., 2013), the alignment of sequences showing variation among individuals within the repeat motif, and the flanking sequences allowing primer design. The criteria for the primer design were as follows: primer length of 18–27 bp, GC content 40–60%, annealing temperature 55–58°C, and the expected amplicon size of 100–300 bp. Primer pairs homologous to the flanking regions were designed for 16 cpSSR loci using Primer3 software (Rozen and Skaletsky, 2000). The primers were obtained from Oligomer Oy (Helsinki, Finland). The forward primers were labeled with fluorescent dyes for automated electrophoresis.

Genomic DNA of *F. rubra* was extracted from fresh leaves using the E.Z.N.A. Plant DNA Kit (Omega Bio-Tek, Norcross, Georgia, USA). The yield and purity of DNA were measured using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA). PCR amplifications were performed in a final volume of 10 μL, containing 5–10 ng genomic DNA, 1× GoTaq Flexi Buffer, 1.0 mM $MgCl_2$ solution, 0.2 mM of each dNTP, 0.2 μM of each primer, and 1.25 units GoTaq G2 HotStart Polymerase (Promega Corporation, Madison, Wisconsin, USA). The PCR reactions were performed as follows: an initial denaturation at 95°C for 2 min; followed by 30 cycles of 30 s at 95°C, 30 s at 56°C, and 30 s at 73°C; and a final extension for 5 min at 73°C, using a C1000 Thermal Cycler (Bio-Rad, Applied Biosystems, Foster City, California, USA). Each microsatellite marker was amplified singly. A set of PCR products, 4 μL per PCR reaction, were checked for amplification success using 1.5% agarose gels (SeaKem LE Agarose; Lonza, Rockland, Maine, USA). The PCR products were run on an ABI 3130xl DNA Sequencer using the GeneScan 500 ROX Size Standard (Applied Biosystems) at the Institute of Biotechnology, University of Helsinki, Finland, and the amplified fragment lengths were assigned to allelic sizes with Peak Scanner version 1 software (Applied Biosystems).

Characteristics of the 16 markers were initially tested by multiplexing markers with different fluorescent labels and expected fragment sizes, and including four samples originating from different geographic regions (Appendix 1). All primer pairs produced bands that matched the expected sizes. The 16 markers were arranged in multiplex sets for genotyping. Markers were screened for polymorphism using 93 samples originating from six different populations located in a wide geographic region, including Finland, Greenland, the Faroe Islands, Norway, and two locations in Spain (Appendix 1). The resulting genotyping data were analyzed using GenAlEx version 6.5 (Peakall and Smouse, 2006, 2012) to estimate the number of alleles per locus and unbiased haploid diversity.

Thirteen out of 16 markers were polymorphic, one marker was monomorphic, and two markers amplified unreliably (Table 1). Thirteen polymorphic markers amplified a total of 65 alleles in a sample of 93 individuals of *F. rubra* originating from the six different populations. The number of alleles per polymorphic locus varied from two to eight at the species level and from one to six at the population level, and the unbiased haploid diversity per locus varied from 0.104 to 0.795 at the species level and from 0.000 to 0.824 at the population level (Table 2). The southern populations from Spain possessed a greater number of alleles and higher haploid diversity compared to the northern populations.

TABLE 2. Characteristics of 13 polymorphic chloroplast microsatellite loci in six populations of *Festuca rubra* within a wide geographic region.[a]

	BERG ($n = 15$)		FAS2 ($n = 15$)		GL1 ($n = 12$)		HA1 ($n = 15$)		SPGD ($n = 18$)		SPPOR ($n = 18$)		All ($n = 93$)	
Locus	*A*	*h*	*A*	*h*	*A*	*h*	*A*	*h*	*A*	*h*	*A*	*h*	*A*	*h*
FR15cpSSR	1	0.000	1	0.000	1	0.000	2	0.343	1	0.000	3	0.216	4	0.104
FR16cpSSR	2	0.514	2	0.248	1	0.000	2	0.133	4	0.471	4	0.542	4	0.370
FR17cpSSR	2	0.514	2	0.514	2	0.167	2	0.133	3	0.582	3	0.386	5	0.609
FR19cpSSR	3	0.590	2	0.133	2	0.167	1	0.000	2	0.294	2	0.425	4	0.471
FR20cpSSR	3	0.676	3	0.590	2	0.167	5	0.733	5	0.824	4	0.778	8	0.795
FR21cpSSR	2	0.533	3	0.257	1	0.000	3	0.257	5	0.693	6	0.784	7	0.596
FR24cpSSR	3	0.600	3	0.590	1	0.000	2	0.533	6	0.797	6	0.824	7	0.722
FR26cpSSR	2	0.514	2	0.514	2	0.167	2	0.133	4	0.732	4	0.725	5	0.601
FR27cpSSR	2	0.514	1	0.000	1	0.000	1	0.000	2	0.294	1	0.000	2	0.177
FR28cpSSR	2	0.248	3	0.705	2	0.167	1	0.000	3	0.582	3	0.582	3	0.571
FR29cpSSR	1	0.000	1	0.000	1	0.000	1	0.000	3	0.601	4	0.608	4	0.300
FR30cpSSR	1	0.000	1	0.000	2	0.167	2	0.133	3	0.529	3	0.660	4	0.320
FR31cpSSR	1	0.000	3	0.648	1	0.000	2	0.133	4	0.686	3	0.582	5	0.455
Mean	1.9	0.362	2.1	0.323	1.5	0.077	2.0	0.195	3.5	0.545	3.5	0.547	4.8	0.469

Note: *A* = number of alleles per locus; *h* = unbiased haploid diversity; *n* = sample size.
[a] Population information is provided in Appendix 1.

CONCLUSIONS

Thirteen novel polymorphic chloroplast microsatellite markers designed for fine-leaved fescues showed a considerable amount of genetic variation within *F. rubra* populations. This set of novel polymorphic cpSSR markers provides a valuable tool for grass breeders, taxonomists, and population geneticists investigating fine-leaved *Festuca* taxa, which presumably cross-amplify, especially within the *F. ovina* and *F. rubra* groups (Inda et al., 2008).

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Appendix 1. Voucher information for *Festuca rubra* specimens used in this study.

Taxon	Population code	Locality	Geographic coordinates	Altitude (m)	Habitat	Voucher specimen[a]
Festuca rubra s.l.	BERG	Kinsarvik, Norway	60°22′43″N, 6°43′32″E	0	Seashore meadow	H1761060
Festuca rubra subsp. *rubra*	FAS2	Vidoy, Faroe Islands	62°22′3.4″N, 6°32′31.8″W	148	Meadow	H1762440
Festuca rubra subsp. *arctica*	GL1	Disko, Greenland	69°14′59″N, 53°31′15″W	1	Sandy seashore	H1757969
Festuca rubra subsp. *rubra*	HA1	Hanko, Finland	59°50′27″N, 23°13′15″E	1	Seashore meadow	H1762441
Festuca cf. *rubra*	SPGD	Cáceres, Spain	40°12′1.12″N, 5°45′11.03″W	768	Xerophytic forest	H1762442
Festuca rubra subsp. *rothmaleri*[b]	SPPOR	Salamanca, Spain	40°58′24.28″N, 5°57′33.69″W	812	Grassland “dehasa”	H1762443

[a] Vouchers deposited at the Botanical Museum (H), University of Helsinki.
[b] This taxon is also treated as the species *Festuca rothmaleri*.