**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 heteroplasmacy, 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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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 (Thornton 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 accession numbers of *F. rubra* intergenic chloroplast microsatellite 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 repetition level (Table 2) and the region located in a wide geographic region, including Finland, Greenland, the Faroe Islands, Norway, and Spain (n = 12–18 for each population); see Appendix 1 for population information.

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 GenAIEx version 6.5 (Peakall and Smouse, 2006, 2012) to estimate the number of alleles per locus and unbiased haploid diversity.

| Locus     | Primer sequences (5′−3′) | Repeat motif | Allele size range (bp) | Position | GenBank accession no. |
|-----------|-------------------------|--------------|------------------------|----------|-----------------------|
| FR15cpSSR | F: CCCTCTCCTCCGGTTCCAAA | (T)12(C)7(T)6 | 203–212 | trn-S-GCU/pbsD | JX871940 |
| R: TGTGCTCCTTGGCATATTGAA | | | | | |
| FR16cpSSR | F: AGGGCCAGATTGATTAAGCCAGGT | (TAT)(T)(TAT)(T)6 | 229–234 | trn-S-GCU/pbsD | JX871940 |
| R: ATGTGGCAGGGTGTGACACT | | | | | |
| FR17cpSSR | F: GGGCGATCACTGAGGATAC | (A)12(C)13 | 217–222 | ycf3/trnS-GGA | JX871940 |
| R: TGCCCAACATCGAGGAAAA | | | | | |
| FR19cpSSR | F: TAAAGCAGCGGTTCTCTCCA | (A)12 | 174–180 | trn-T-UGU/trnL-UAA | JX871940 |
| R: AACAATAGGGTTACGGCTGCT | | | | | |
| FR20cpSSR | F: TCTCGGTGTCAGCAGTTCAA | (A)12(A)3(A)7 | 245–257 | ntrF-GAA/nhj | JX871940 |
| R: AGGGTATATTCCCTGCTCGTC | | | | | |
| FR21cpSSR | F: AGGACTAATCTGGCTGTAATAGGAA | (A)12(G)3(A)16(T)7 | 246–260 | ndhC/trnV-UAC | JX871940 |
| R: TCCATTTGGCGATTACCCTTG | | | | | |
| FR23cpSSR | F: TCCACTTTCTTTATCGCTCTGT | (A)16(T)7 | 182 | pbsE/petL | JX871940 |
| R: AGGCGGAGTTAGAAGACCGA | | | | | |
| FR24cpSSR | F: CCGTCTTATATAGGGGATAGGCT | (AT)12(AT)6(AT)3(AT)3(AT)3 | 292–301 | ndhF/rpl32 | JX871940 |
| R: GTCTGCAATAACTCTCCTCCT | | | | | |
| FR26cpSSR | F: AGTCCGCTCTGCCCTCCT | (T)12(T)6 | 186–190 | atp1/atpH | JX871940 |
| R: TGTGACATTGGCAGTTAG | | | | | |
| FR27cpSSR | F: GAGGAATGTGGCAGTTTCTT | (T)7(C)(T)6(TT)4 | 198–201 | petA/pbsJ | JX871940 |
| R: TACCTGCCGCTGACTACCGC | | | | | |
| FR28cpSSR | F: AGGAGAACAGAAGCTATGACAGA | (A)11 | 122–124 | trn-T/trnL | EF585096 |
| R: CTTCCTGCCGCCGCCCTATTTA | | | | | |
| FR29cpSSR | F: TCAATTGTATATGCTGCTAGAGGA | (AT)(A)13 | 191–200 | trn-T/trnL | DQ336857.1 |
| R: TGTGATAGGCTGTGTTCTCCTC | | | | | |
| FR30cpSSR | F: CAGCATAATGGTGTCTGTGCC | (T)(T)7(C)(A)(T)4 | 226–231 | rps8/ppl14 | HMI73006 |
| R: GATTCGCCAGAATGTGAGAGGA | | | | | |
| FR31cpSSR | F: TGCAGAAGAGGTGCTGAGAGAG | (C)8 | 250–254 | trn-Lm/trnF | EF593001 |
| R: CTGTTGACATCTGCTATTAGAGT | | | | | |

*Annealing temperature = 56°C. *

*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.*

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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 |
|---------------------------|-----------------|------------------------|--------------------------|--------------|------------------|------------------|
| *Festuca rubra* s.l.      | BERG            | Kinsarvik, Norway      | 60°22'43"N, 6°43'32"E   | 0            | Seashore meadow  | H1761060         |
| *Festuca rubra* subsp.    | FAS2            | Vidoy, Faroe Islands   | 62°22'3.4"N, 6°32'31.8"W | 148          | Meadow           | H1762440         |
| *Festuca rubra* subsp.    | GL1             | Disko, Greenland       | 69°14'59"N, 53°31'15"W   | 1            | Sandy seashore   | H1757969         |
| *Festuca rubra* subsp.    | HA1             | Hanko, Finland         | 59°50'27"N, 23°13'15"E   | 1            | Seashore meadow  | H1762441         |
| *Festuca rubra* subsp.    | SPGD            | Cáceres, Spain         | 40°12'1.12"N, 5°45'11.03"W | 768          | Xerophytic forest| H1762442         |
| *Festuca rubra* subsp.    | 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*.  

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