Development and Characterization of Nuclear Microsatellite Markers in the Endophytic Fungus Epichloë festucae (Clavicipitaceae)

Authors: Cräutlein, Maria von, Korpelainen, Helena, Helander, Marjo, Öhberg, Annika, and Saikkonen, Kari

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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF NUCLEAR MICROSATELLITE MARKERS IN THE ENDOPHYTIC FUNGUS *EPICHLOË FESTUCAE* (CLAVICIPITACEAE)[1]

MARIA VON CRÄUTLEIN[2,5], HELENA KORPELAINEN[3], MARJO HELANDER[2,4], ANNIKA ÖHBERG[3], AND KARI SAIKKONEN[2]

[2]MTT Agrifood Research Finland, Plant Production Research, Tietotie, 31600 Jokioinen, Finland; [3]Department of Agricultural Sciences, University of Helsinki, Latokartanonkaari 5, FI-00014 University of Helsinki, Finland; and [4]Department of Biology, University of Turku, 20014 Turku, Finland

- *Premise of the study:* Microsatellite primers were developed for the endophytic fungus *Epichloë festucae*, which is symbiotic with *Festuca rubra*, to study the population genetics of the species and to compare population structures between *E. festucae* and its host *F. rubra.*
- *Methods and Results:* We developed 14 polymorphic markers using the unplaced genomic scaffold sequences of *E. festucae* from GenBank. The number of alleles per locus (*A*) varied from four to 16, and unbiased haploid diversity (*h*) was 0.717 in eight populations located in the Faroe Islands, Finland, and Spain. The Spanish populations possessed a higher number of alleles and haploid diversity (on average $A = 5.1$ and $h = 0.591$, respectively) compared to northern populations (on average $A = 1.5$ and $h = 0.199$, respectively).
- *Conclusions:* These polymorphic markers will be used by grass breeders for uses including the improvement of commercial turfgrass cultivars, and by population geneticists to study different species of the *Epichloë* genus.

Key words: Ascomycota; Clavicipitaceae; endophyte; *Epichloë festucae*; *Festuca rubra*; fungus; grass.

Endophytic fungi are very common and important components in plant microbiomes. They are capable of infecting their host plant's tissues without causing obvious symptoms to the host (Hyde and Soytong, 2008). The *Epichloë* endophytes (Ascomycota: Clavicipitaceae) are one of the most studied systems of plant-endophyte associations because they form symbiotic relationships with several economically important turf and forage cool season grasses (Clay and Schardl, 2002). These endophytes are important agents influencing the growth and persistence of host grasses, and a genetically compatible endophyte infection has been demonstrated to provide a selective advantage to the host (Ahlholm et al., 2002; Saikkonen et al., 2006, 2010a, b). The *Epichloë* endophytes are a group of filamentous fungi that comprise sexual *Epichloë* species and their asexual derivatives, *Neotyphodium* species, which have been recently classified as *Epichloë* (Leuchtmann et al., 2014). *Epichloë festucae* Leuchtm., Schardl & Siegel is a fungal epichloid systemic endophyte, which systematically and intercellularly colonizes aboveground tissues and seeds of *Festuca rubra* L. by means of haploid hyphae. *Festuca rubra* is a perennial grass with rapid expansion worldwide in a wide range of ecosystems, and it is one of the most important turfgrasses in temperate regions (Inda et al., 2008). Previously, nuclear microsatellite markers have been developed for *Epichloë* species by Moon et al. (1999) and expressed sequence tag (EST)–derived simple sequence repeats (SSRs) for pasture grass endophytes by van Zijll de Jong et al. (2003), and among those markers, four polymorphic nuclear microsatellite markers have been used in a population genetic study on *E. festucae* (Wäli et al., 2007). Because highly polymorphic genomic microsatellites are effective tools for studying population genetic characteristics, in this study, we aim to develop additional polymorphic microsatellite primers for *E. festucae*.

METHODS AND RESULTS

The unplaced genomic scaffold sequences of *E. festucae* were downloaded (GenBank accession no. JH158803–JH158837) and searched for ≥10 mono- and dinucleotide repeats, and for ≥8 tri-, tetra-, penta-, and hexanucleotide repeats by using MSATCOMMANDER (Faircloth, 2008). The selected marker regions possessed the maximum length of the repeat motif, a minimum distance of 100,000 bp between the repeat motifs within the same accession, and the presence of appropriate flanking sequences for primer design with the following criteria: primer length of 18–27 bp, GC content 40–60%, annealing temperature 55–58°C, and expected amplicon size of 100–300 bp. Twenty-four primer pairs were designed with Primer3 software (Rozen and Skaletsky, 2000). The forward primers were labeled with fluorescent dyes for automated electrophoresis, and the primers were obtained from Oligomer Oy (Helsinki, Finland).

To isolate *E. festucae* from endophyte-infected (E+) plants of *F. rubra*, three leaves were collected from each selected tiller from pots containing replanted *F. rubra* in the greenhouse of Ruissalo Botanical Garden, Finland. The plant material was kept at 4°C for 24 h, followed by surface sterilization, including a treatment in 75% ethanol for 30 s, 4% sodium hypochlorite for 3 min, and 75%

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[5]Author for correspondence: maria.voncrautlein@helsinki.fi

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TABLE 1. Characteristics of the 15 nuclear microsatellite markers developed for the endophyte fungus *Epichloë festucae*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Starting position (bp)	Allele size range (bp)[b]	GenBank accession no.
EF1SSR	F: 6FAM-ATCTGATTTGAAACCCGGCG R: AGCTCTCTTTCTCCCCACAC	$(A)_{36}(G)_8$	92,753	169–189	AFRX01000154
EF2SSR	F: 6FAM-GCGTTTTGGGCAGGTTATGT R: GGTCACGTCATACTGAGTGC	$(AT)_{12}(GT)_{21}$	45,291	144–168	AFRX01000490_3
EF4SSR	F: HEX-TGACTCAAGAAACGGTGCAG R: GCAGCGAACTTCCATGTGTA	$(CT)_{20}$	97,543	198–226	AFRX01000056_1
EF5SSR	F: 6FAM-TCATACGGATTAGTGGCCCC R: GTCAACTTTTCATTCCGTCCCT	$(AC)_{17}$	54,068	217–351	AFRX01000491
EF6SSR	F: HEX-ACACTTGCCTTTTGGAGCAT R: GCGCTCAACTTCGTCTCTCT	$(AG)_{16}$	54,068	218–250	AFRX01000295
EF7SSR	F: 6FAM-GGCACAAAGGAACAGGACAT R: AGCAATTGTTCGGGAATCAG	$(TA)_{15}$	125,913	94–104	AFRX01000004_4
EF10SSR	F: 6FAM-ACTCTGACGGGCTGACACTC R: AAGGGAAAAAGCGAAGAAGC	$(GTT)_8(CCT)_{18}$	104,071	224–228	AFRX01000322_2
EF12SSR	F: HEX-TGCTCAACCATTTCTTCGTG R: GGGCAAACATCAGTTCGATT	$(CGA)_{13}$	2653	152	AFRX01000322_2
EF14SSR	F: 6FAM-ATTAGGTTTGGCAGCCGATG R: TGGTGAAACAACCCCGGAAT	$(AGG)_{11},(GGT)_7$	19,493	212–234	AFRX01000027_1
EF15SSR	F: HEX-CACAGGTGTGCCTTGTCAAC R: AACGGGAGTGAGACAGCATC	$(CAT)_8TAT(CAT)_6$	21,195	215–237	AFRX01000059_2
EF19SSR	F: HEX-TGCAGGTCTCGTCTTCTCTC R: GGACGATGCAAATGACTGACA	$(GCCT)_{13}$	14,731	198–319	AFRX01000166
EF20SSR	F: 6FAM-TTGAGTACAGGACAGGACGG R: CGCTCAGATGTTGGATGACG	$(AAGC)_{13}$	10,240	164–188	AFRX01000153
EF21SSR	F: HEX-GATTAGACACGACGCGGAAG R: CCCTGTCGTTATGGACTCGT	$(CATT)_{12}$	84,927	130–312	AFRX01000271
EF22SSR	F: HEX-GCAATCCCAAAACATGACGC R: GCAAAACATGTGAAACGGCC	$(GAGT)_{12}$	49,308	103–258	AFRX01000490_2
EF24SSR	F: HEX-CCCGAGTACTATGGTGGCAA R: CGACTTCCATGCACACTGTT	$(ACTCTC)_9$	98,200	197–258	AFRX01000156

[a]Annealing temperature = 57°C.

[b]The size ranges (bp) are based on 70 samples representing European populations located in Finland, the Faroe Islands, and Spain (N = 8–10 for each population); see Appendix 1 for population information.

ethanol for 15 s. Then, a leaf was cut into five segments and planted on autoclaved Petri dishes containing 5% potato dextrose agar (PDA). Agar plates were stored at room temperature until mycelium emerged from the plated leaf fragments, after which a small sample of mycelium was transferred to a new PDA plate on a piece of sterile cellophane (9 cm in diameter). *Epichloë festucae* identity was determined based on the morphological characteristics observed in cultures, and based on the sequences of the ITS1, 5.8S rRNA, and ITS2 region of a set of isolates from different locations, compared with GenBank resources (http://www.ncbi.nlm.nih.gov) using BLAST searches. PCR amplification of the ITS1, 5.8S rRNA, and ITS2 region was performed using primers ITS1 and ITS5 (http://www.fungalbarcoding.org/). *Epichloë festucae* is the only systemic fungus described for *F. rubra*, and the systemic endophyte has always been *E. festucae* when we have previously sequenced fungi from *F. rubra* (e.g., Wäli et al., 2007). The risk that the mycelium growing on PDA corresponds to another related fungal endophyte different from *E. festucae* is marginal, especially as grasses are commonly assumed to be infected by only one systemic fungus. Replanted agar plates were stored at room temperature until the growth of the mycelium was sufficient for DNA extraction. Mycelium growth was

TABLE 2. Characteristics of 14 nuclear polymorphic microsatellite loci in eight populations of *Epichloë festucae*.[a]

	FAS1 (n = 8)		FAS2 (n = 8)		FAS5 (n = 8)		FAS6 (n = 8)		RBS1 (n = 8)		SPGD (n = 10)		SPLV (n = 10)		SPPOR (n = 10)		All (n = 70)	
Locus	A	h	A	h	A	h	A	h	A	h	A	h	A	h	A	h	A	h
EF1SSR	1	0.000	1	0.000	1	0.000	1	0.000	3	0.607	2	0.533	2	0.533	2	0.200	6	0.700
EF2SSR	1	0.000	1	0.000	1	0.000	2	0.250	1	0.000	3	0.733	3	0.600	2	0.467	6	0.727
EF4SSR	1	0.000	1	0.000	1	0.000	1	0.000	1	0.000	2	0.556	4	0.778	1	0.000	7	0.706
EF5SSR	3	0.679	2	0.536	2	0.536	2	0.571	2	0.250	4	0.800	3	0.511	3	0.378	9	0.838
EF6SSR	2	0.250	1	0.000	2	0.250	1	0.000	1	0.000	6	0.889	3	0.622	3	0.511	12	0.749
EF7SSR	1	0.000	1	0.000	1	0.000	1	0.000	1	0.000	3	0.600	2	0.200	2	0.556	4	0.598
EF10SSR	1	0.000	1	0.000	1	0.000	1	0.000	1	0.000	2	0.467	3	0.644	3	0.511	5	0.702
EF14SSR	2	0.250	1	0.000	1	0.000	3	0.714	1	0.000	3	0.511	3	0.733	4	0.711	8	0.767
EF15SSR	1	0.000	1	0.000	1	0.000	2	0.250	1	0.000	2	0.356	4	0.778	1	0.000	7	0.436
EF19SSR	2	0.536	2	0.571	2	0.536	3	0.679	1	0.000	4	0.711	5	0.800	4	0.733	14	0.874
EF20SSR	1	0.000	2	0.571	2	0.429	1	0.000	1	0.000	2	0.200	3	0.733	2	0.556	5	0.435
EF21SSR	2	0.536	2	0.429	2	0.536	2	0.536	3	0.607	7	0.911	6	0.778	5	0.844	16	0.868
EF22SSR	2	0.429	3	0.679	3	0.464	1	0.000	2	0.429	4	0.533	6	0.867	7	0.933	15	0.861
EF24SSR	2	0.250	2	0.571	1	0.000	1	0.000	1	0.000	4	0.778	3	0.600	3	0.644	9	0.779
Mean	1.6	0.209	1.5	0.240	1.5	0.196	1.6	0.214	1.4	0.135	3.4	0.613	3.6	0.656	3.0	0.503	8.8	0.717

Note: A = number of alleles per locus; h = unbiased haploid diversity; n = sample size.

[a]Geographic coordinates for the populations are provided in Appendix 1.

scraped from the cellophane into an Eppendorf tube for DNA extraction. DNA was extracted from pure cultures of *E. festucae* with the E.Z.N.A. Plant DNA Kit (Omega Bio-Tek, Norcross, Georgia, USA), and a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA) was used to reveal the yield and purity of DNA. The PCR reactions were performed with a single microsatellite primer pair in a 10-µL reaction mixture 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). PCR reactions were performed in a C1000 Thermal Cycler (Bio-Rad, Applied Biosystems, Foster City, California, USA) as follows: an initial denaturation at 95°C for 2 min; followed by 30 cycles at 95°C for 30 s, 57°C for 30 s, and 73°C for 30 s; and a final extension at 73°C for 5 min. Each PCR product was amplified singly. The amplification success was controlled with a set of PCR products using 2% agarose gels (SeaKem LE Agarose; Lonza, Rockland, Maine, USA). The products were run on an ABI 3130xl Genetic Analyzer using the GeneScan 500 ROX Size Standard (Applied Biosystems) at the Institute of Biotechnology, University of Helsinki, Finland, and assigned to allelic sizes with Peak Scanner version 1 software (Applied Biosystems). The unbiased haploid diversity (h) and the number of alleles (A) per locus and population were calculated using GenAlEx version 6.5 (Peakall and Smouse, 2006, 2012).

Initially, 24 individuals originating from populations in the Faroe Islands ($N = 8$), Finland ($N = 8$), and Spain ($N = 8$) were screened to reveal the competence of the 24 primer pairs. Nine primer pairs out of 24 were rejected from the further analysis because of unclear patterns with multiple bands and allelic dropouts, whereas 15 primer pairs amplified reliably and produced clearly interpretable single bands, and these were used in the further analyses (Table 1). Fifteen loci were screened for polymorphism using 70 individuals originating from four different populations in the Faroe Islands, one population from Finland, and three populations from Spain (Tables 1 and 2). One locus was monomorphic while 14 loci revealed polymorphism with altogether 123 alleles (Table 1). The number of alleles per locus varied from four to 16 at the species level and from one to seven at the population level (Table 2). The unbiased haploid diversity per locus varied from 0.435 to 0.874 at the species level, and from 0.000 to 0.933 at the population level (Table 2). The Spanish populations possessed a considerably higher number of alleles and haploid diversity (on average $A = 5.1$ and $h = 0.591$, respectively) compared to northern populations (on average $A = 1.5$ and $h = 0.199$, respectively).

CONCLUSIONS

Because endophytes have both scientific relevance and applied importance, these new polymorphic microsatellite markers will be useful for grass breeders, e.g., to improve commercial turfgrass cultivars of *F. rubra*, and for researchers to study different aspects of grass endophyte evolution. These markers presumably cross-amplify within the genus *Epichloë*, which includes host-specific endophytes of several important forage grasses (Leuchtmann et al., 2014).

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Appendix 1. Voucher information for *Epichloë festucae* isolates used in this study.

Population code	Locality	Geographic coordinates	Altitude (m)	Habitat	Voucher specimen of host plant[a]
FAS1	Mykines, Faroe Islands	62°5′50.7″N, 7°40′55.9″W	125	Meadow	H1762448
FAS2	Vidoy, Faroe Islands	62°22′3.4″N, 6°32′31.8″W	148	Meadow	H1762445
FAS5	Vagar, Faroe Islands	62°6′58.6″N, 7°26′42.5″W	246	Meadow	H1763054
FAS6	Eysturoy, Faroe Islands	62°17′24.4″N, 7°2′9.7″W	316	Meadow	H1762447
RBS1	Kevo, Finland	69°54′35.1″N, 27°2′0.15″E	73	Riverbank	H1762450
SPGD	Cáceres, Spain	40°12′1.12″N, 5°45′11.03″W	768	Xerophytic forest	H1762444
SPLV	Salamanca, Spain	40°56′20.16″N, 6°7′6.60″W	863	Grassland “dehasa”	H1762449
SPPOR	Salamanca, Spain	40°58′24.28″N, 5°57′33.69″W	812	Grassland “dehasa”	H1762446

[a]Vouchers deposited at the Botanical Museum (H), University of Helsinki.