Eur J Plant Pathol (2009) 123:477–482
DOI 10.1007/s10658-008-9379-x

Characterisation of novel *Fusarium graminearum* microsatellite markers in different *Fusarium* species from various countries

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Received: 15 July 2008 / Accepted: 29 September 2008 / Published online: 1 November 2008

Abstract Fifteen novel microsatellite markers were isolated from *Fusarium graminearum*. The level of polymorphism at these novel and 13 previously published microsatellite markers was analysed in 33 *F. graminearum* strains from Europe, North America, and Nepal. The number of alleles for each of the novel markers ranged from 4 to 20 and gene diversity from 0.417 to 0.962. In comparison with the previously published markers, the resolution for distinguishing among different strains was slightly increased. Twenty-seven markers were also detectable in three *F. culmorum* strains and one *F. crookwellense* strain. None of the markers was detected in three *F. avenaceum* and four *F. poae* strains, underlining the potential use of these microsatellite markers for species differentiation.

Keywords Cereals · Fusarium head blight · Genetic diversity · Simple sequence repeat

Fusarium head blight (FHB) is one of the most noxious fungal cereal diseases worldwide, resulting in severe yield losses and a decline in cereal quality (Goswami and Kistler 2004). FHB can be caused by several *Fusarium* species. In Europe for instance, the predominant FHB pathogens are *F. graminearum*, *F. culmorum*, *F. avenaceum*, and *F. poae* (Nicholson et al. 2003). On a global scale, *F. graminearum* is the most prevalent FHB-causing species (Gale 2003). Analysis of sequence signatures in eight genes has suggested that *F. graminearum* may consist of several distinct phylogenetic lineages with similar morphology and pathogenicity and it has been proposed that these lineages may represent distinct species (O'Donnell et al. 2000, 2004). However, the validity of this species delimitation has been questioned and it has been suggested that some of the lineages represent geographically separated and genetically distinct populations rather than distinct species (e.g. Jurgenson et al. 2002; Zeller et al. 2004; Miedaner et al. 2008). For example, various fertile crosses between different lineages have been demonstrated (Bowden et al. 2006). Furthermore, sequence data of one (*TRI101*) out of the eight genes mentioned above in ~500 strains revealed sequence signatures that could not be assigned to the proposed lineages (Leslie et al. 2007). Due to this unresolved taxonomy, we refer here to *F. graminearum sensu lato*, considering the entire *F. graminearum* complex as a single species.

Various molecular genetic tools have been developed for population genetic analysis of *F. graminearum* and *F. culmorum*. They include analyses of amplified fragment length polymorphisms (AFLP), restriction fragment length polymorphisms (RFLP) (summarised by Gale 2003), as well as mini- (Suga et al. 2004) and microsatellite markers (Giraud et al. 2002; Naef et al. 2006; Naef and Défago 2006).

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Table 1 *Fusarium* spp. strains used in this study

Species and strain name[a]	Geographic origin
F. graminearum	
CBS 121291[b]	Switzerland
CBS 121292[b]	Switzerland
Fg0366[b]	Switzerland
Fg0370[b]	Switzerland
Fg0371[b]	Switzerland
Fg0406[b]	Switzerland
Fg0407[b]	Switzerland
Fg0408[b]	Switzerland
Fg0411[b]	Switzerland
Fg0412[b]	Switzerland
PH-1 (NRRL 31084)[c]	USA
Z-3635[d]	USA
Z-3636[d]	USA
Z-3639[e]	USA
Z-3654[d]	USA
DAOM 178148[f]	Canada
DAOM 180378[f]	Canada
IBT 1958[g]	Denmark
IBT 40042[g]	Denmark
6473[h]	Italy
633[h]	Italy
GGM 87[i]	Nepal
GGM 93[j]	Nepal
FG 0433D[j]	Norway
11669[j]	Norway
L1–2/1D[k]	Spain
L3–1/3J[k]	Spain
05113[l]	Finland
DSMZ 4528[m]	Germany
HUGR2[n]	Hungary
L18C[n]	Ireland
CBS 115697[o]	Poland
CBS 389.62[o]	The Netherlands
F. culmorum	
Fc9712[b]	Switzerland
Fc9714[b]	Switzerland
Fc9716[b]	Switzerland
F. crookwellense	
CBS 121293[b]	Switzerland
F. avenaceum	
CBS 121289[b]	Switzerland
CBS 121290[b]	Switzerland
CBS 121294[b]	Switzerland
F. poae	
CBS 121298[b]	Switzerland
CBS 121299[b]	Switzerland
IBT 2926[g]	Denmark
CBS 186.96[o]	Poland

[a] Strains were obtained from the following institutions:

[b] Agroscope Reckenholz-Tänikon, Switzerland

[c] Fungal Genetics Stock Center, Kansas City, MO, USA, Kevin McCluskey

[d] USDA-ARS, Manhattan, KS, USA, Robert Bowden

[e] USDA-ARS, NCAUR, Peoria, IL, USA, Robert Proctor

[f] Canadian Collection of Fungal Cultures, Ottawa, Canada, Keith Seifert

[g] BioCentrum-DTU, Lyngby, Denmark, Ulf Thrane

[h] Istituto di Scienze delle Produzioni Alimentari, Bari, Italy, Antonio Moretti

[i] USDA-ARS, NCAUR, Peoria, IL, USA, Anne Desjardins

[j] Bioforsk, Plant Health and Protection Division, Ås, Norway, Birgitte Henriksen

[k] Departamento de Genetica, Universidad Complutense de Madrid, Spain, Maria T. G. Jaen

[l] MTT Agrifood Research, Jokioinen, Finland, Marja Jalli

[m] DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany

[n] School of Biology & Environmental Science, University College, Dublin, Ireland, Mike Cooke

[o] Fungal Biodiversity Centre, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands

The aim of this study was to develop novel microsatellite markers for *F. graminearum* in order to elucidate whether it is possible to increase the resolution of existing markers to discriminate between individual strains or whether saturation of discrimination has been reached. This information is of fundamental importance when using microsatellites in future studies on the pathogenicity, ecology, or taxonomy of this species. In order to approach this question, novel and previously published microsatellite markers were compared using 33 strains of *F. graminearum*. Furthermore, these markers were also tested for their ability to differentiate *F. graminearum* from other *Fusarium* species.

Thirty-three single-conidium strains from *F. graminearum* originating from 14 countries were used for this study (Table 1). Strain PH-1 (NRRL 31084), from which the genome has been fully sequenced, served as the reference strain for microsatellite marker design. To test for cross-species amplification, three

Table 2 Primer sequences and characteristics of 15 novel *Fusarium graminearum* and *Fusarium culmorum* microsatellite markers

Name	Contig[a]	T_a (°C)[b]	Reference strain[c]		PCR primers (5′-3′)[d]	*F. graminearum*			*F. culmorum*	
			Fragment size (bp)	Repeat		No. alleles	Allelic size range (bp)	Gene diversity[e]	No. alleles	Allelic size range (bp)
FusSSR12	1.168	65	281	$(GCT)_{10}$	[f]ATTAGAGAGTGCGGCAGAAAGAG CAGACATGTTCAAGGGCAATTG	7	263–281	0.653	1	260
FusSSR13	1.354	65	201	$(GCT)_{15}$	[f]CGACTCAGATTGATTGCTGCC CCCTTCTCCGATTAAACCACAAC	6	175–238	0.733	1	163
FusSSR14	1.12	65	254	$(GA)_{18}$	[f]TTCAGCATCACTTGCGTCG TACCGCCCCTCACTCTTATACC	8	237–261	0.769	2	251–253
FusSSR16	1.212	65	166	$(TG)_{19}$	[f]CTTTCTCCAACCGAATTGGG AGTACGGACCGACGACATAACTC	12	139–195	0.845	1	145
FusSSR17	1.224	65	221	$(GA)_{19}$	[f]AATCCGGTATCCGTACAACCC AAGAACTACTTCGCTGCCGTG	14	203–245	0.837	1	199
FusSSR18	1.237	68	401	$(GAA)_{15}$	[f]GAATACTCAAGTCCCGTAGCCG TGCAGAGACCCATAAACGACG	12	380–418	0.892	1	410
FusSSR19	1.24	65	201	$(GATA)_8$	[g]AGCCGGACATGAGACAAAGTAG TGTTGTTCCCTCCAGTACTCG	5	184–204	0.688	1	188
FusSSR20	1.262	65	208	$(TG)_{12}$ TA $(TG)_{24}$	[g]GCTGTTAGCTTGCAGGACAGC AAGAATATTTCCACGCAACTTGG	20	139–235	0.962	1	129
FusSSR21	1.292	68	166	$(AGC)_7$	[g]TCTTGACTGCCCAACATGGAC GGGCAATATGAAGTCTGCGTC	7	154–175	0.652	2	163–169
FusSSR22	1.32	68	211	$(GAT)_{10}$	[g]GAGGGCGATGGTTGAAGTGTAC TGGGCATGAAACAAGAGAGAGAC	4	200–212	0.417	1	200
FusSSR23	1.34	65	201	$(CT)_{28}$	[g]GTTGACACAGAAGAATGGCAGG CGCTAGGTACAAATTGCTGGG	19	165–221	0.951	3	137–165
FusSSR24	1.352	65	151	$(CA)_{19}$	[g]CGGACATCTTCACCTGGTCC GCCGCGAATACAGAGTGCTC	7	122–152	0.545	3	126–130
FusSSR26	1.401	68	156	$(GTT)_8$	[g]CTAAGTTGCCAATGGACGTCAG AGTTTTCTGCAGCCCCAAGAG	5	145–157	0.661	1	148
FusSSR27	1.434	65	201	$(CA)_{22}$	[g]TCACCAAAAGTCTCCTCAGTCAAC GTGGTCTCCGTAAACGAGCC	12	157–205	0.858	1	169
FusSSR28	1.46	65	115	$(CAA)_{13}$	[g]TCATGAGAGATGGGCTTTCTTG AATAATCGTCAGGCATCATCCC	12	80–138	0.873	1	102

[a] Contig number in data base (Anonymous 2005)

[b] Annealing temperature for PCR

[c] PH-1 (NRRL 31084), see Table 1

[d] Upper line 5′-labelled forward primer and lower line reverse primer, respectively

[e] According to Nei (1987)

[f] Hex

[g] Ned

strains of *F. culmorum*, one strain of *F. crookwellense*, three strains of *F. avenaceum*, and four strains of *F. poae* were included in the analysis. All 44 *Fusarium* spp. strains originated from cereal hosts. Mycelium was grown in 200 ml Erlenmeyer flasks containing 1.5% malt extract (L39, Oxoid Ltd., Basingstoke, UK) on a shaker (150 rpm) at room temperature (21± 1°C) in the dark. After 2 to 3 days, the mycelium was harvested by filtration through filter paper (No. 595, Schleicher & Schuell, Feldbach, Switzerland), washed with deionised water and lyophilised. Genomic DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's protocols. Quality of genomic DNA was

Table 3 Primer sequences and characteristics of 13 previously published *Fusarium graminearum* and *Fusarium culmorum* microsatellite markers

Name	T_a (°C)[a]	Repeat of cloned allele[b]	PCR primers (5′-3′)[c]	*F. graminearum*			*F. culmorum*	
				No. alleles	Allelic size range (bp)	Gene diversity[d]	No. alleles	Allelic size range (bp)
F1[e]	60	$(TG)_8$	[i]GACAAGCAAGCGATAGGAAA CTTGATAGCACGGACCGACG	12	176–232	0.845	1	182
F3[e]	60	$(CA)_{11}$	[i]CATATTCAACCGACCCACAA TTGAATGATAAGGGCGACGG	5	191–219	0.602	3	197–223
F4[e]	60	$(GT)_{11}$	[i]CTTTTTCCCGGCTCCATTTT GCTTTCCCTGCTCGATCGGG	1	115	–	1	131
F6[e]	60	$(AC)_{15}$	[i]TATTTCGTGCAAGGACTTGG CTTGGTCCCTGGATATCGAA	3	107–135	0.119	3	109–117
F7[e]	60	$(GT)_7$	[i]TGACAAGCAAGCGATAGGAA GAGTGGAGTTTCGATATCGC	12	220–276	0.845	1	228
F11[e]	65	$(GT)_9$	[i]CAGTCTTGGTCGCTCATCAG CAGGTTGGCACGCTTCTTAA	12	294–346	0.858	1	312
MS-Fg103[f]	65	$(TG)_{19}$	[i]GGTATCCGTACAACCCGATG TTCTTTGATTTGGACCGAGG	14	225–267	0.837	1	221
HK917[g]	65	$(CA)_{16}$	[i]ATCTCCCAAGCTGGCTAATT AGAACCGGCAAAGTTCGATT	11	222–266	0.875	0	–
MS-Fc1[h]	60	$(TC)_{17}$	[i]AAGATACCAGGCCTTGATGG AGACTGCATGATTGTTAGCGAG	4	136–144	0.574	2	146–154
MS-Fg30[h]	65	$(AG)_{14}$	[i]TATGACTGCACGTTTGCTCC AGTCTCTAGTGTCGCCGAGG	8	184–216	0.767	1	176
MS-Fg98[h]	60	$(GCAA)_6$	[k]TCTAGGCCAAACCCAGACAC TGTTGCGTTCCTGAAAACAG	4	224–240	0.646	1	220
MS-Fg97[h]	65	$(CCTA)_8$	[k]ACCACTTTTGGTTACAGGCG CCCTTTACTCAAGCTCACGC	8	216–264	0.708	1	208
MS-Fg75[h]	65	$(GAAA)_{11}$	[k]TTGGTATGAAAGACTGCCCC GAAGCGCAAAAGGTATGCTC	17	196–350	0.953	3	206–252

[a] Annealing temperature for PCR

[b] Reference strains: for markers F1-F11: *F. culmorum* L2; all other markers: PH-1 (NRRL 31084)

[c] Upper line 5′-labelled forward primer and lower line reverse primer, respectively

[d] According to Nei (1987)

[e] Giraud et al. (2002)

[f] Naef et al. (2006)

[g] Suga et al. (2004)

[h] Naef and Défago (2006)

[i] Fam

[k] Hex

verified by electrophoresis in 1% agarose and quantified using GelDoc XRS (Bio-Rad Laboratories, Hercules, CA, USA) with Quantity One imaging software (Bio-Rad Laboratories) and the high mass DNA ladder as concentration standard (Promega, Madison, WI, USA).

BLAST similarity searches (Altschul et al. 1990) were performed with different microsatellite motifs in the *F. graminearum* genome database (Anonymous 2005). PCR primer pairs were designed for 15 microsatellite markers using the Primer Express software (Applied Biosystems, Foster City, CA, USA) (Table 2). In addition, 13 previously published markers were included in this study (Table 3). PCR was performed in a volume of 20 µl containing 10 ng template DNA, 3 mM $MgCl_2$, 0.2 mM of each dNTP,

0.2 μM forward (fluorescently labelled, Tables 2 and 3) and reverse primer each (Microsynth, Balgach Switzerland), and 0.5 U of recombinant Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). Cycling conditions consisted of 1 min initial denaturation at 95°C, followed by 36 cycles of 30 s denaturation at 92°C, 30 s annealing at T_a (Tables 2 and 3), and 30 s extension at 72°C. PCR was completed with a 10 min final extension at 72°C. Size determination of amplification products was performed on an ABI Prism 3130XL Genetic Analyser (Applied Biosystems) equipped with 36 cm capillaries and POP-7 polymer. Data were analysed with the Genemarker Software Version 1.51 (SoftGenetics, State College, PA, USA). Gene diversity (Nei 1987) and standard diversity indices were calculated using Arlequin 3.11 (Excoiffier et al. 2005).

All 28 markers were successfully amplified from all 33 *F. graminearum* strains and only marker F4 was not polymorphic, which is in contrast to the findings of Giraud et al. (2002) (Tables 2 and 3). The number of detected alleles for each marker across all *F. graminearum* strains for the novel and the previously published markers ranged from 4 to 20 and 3 to 17 with an average of 10.0 and 8.5, respectively (Tables 2 and 3). Average gene diversity for the novel and the previously published markers was 0.756 (ranging from 0.417 to 0.962) and 0.664 (ranging from 0.119 to 0.953) respectively, indicating that the novel markers detected a slightly higher genetic diversity. Out of the 28 microsatellite markers, only HK917 was not amplified from three *F. culmorum* strains (Tables 2 and 3) and one *F. crookwellense* strain (data not shown). None of the microsatellites could be detected in any of the three *F. avenaceum* or four *F. poae* strains. This result is in line with the phylogeny of FHB species, which associates *F. graminearum* closer to *F. culmorum* and *F. crookwellense* than to *F. avenaceum* and *F. poae* (Kristensen et al. 2005).

Among the novel microsatellite markers, FusSSR28 allowed us to distinguish two more Swiss *F. graminearum* strains, i.e. Fg0407 and CBS 121292, which could not be distinguished with the previously published set of microsatellite markers. The Swiss strains CBS 121291 and Fg0371 as well as the North American strains Z-3639 and DAOM 180378 could not be distinguished with any of the 28 microsatellite markers. Considering that *F. graminearum* strains from various close and distant geographic regions were tested, this finding demonstrates that the resolution for differentiation between strains was merely increased with the novel markers. This indicates that, for *F. graminearum*, the assessment of diversity with microsatellite markers is close to saturation.

Marker F4 revealed species-specific allele sizes, i.e. 115 bp for *F. graminearum*, 131 bp for *F. culmorum* (Table 3), and 121 bp for *F. crookwellense* (data not shown). Among the 27 markers, which were amplified from *F. graminearum* as well as from *F. culmorum,* six previously published (F4, MS-Fg103, MS-Fc1, MS-Fg30, MS-Fg98, and MS-Fg97) and four novel markers (FusSSRs 12, 13, 17, and 20) had no overlapping allelic size ranges between these two species. Therefore, these microsatellites hold potential for distinction of these three FHB species. However, more strains of *F. crookwellense* and *F. culmorum* need to be analysed in order to validate species-specificity of these markers.

This one-to-one comparison of all microsatellite markers currently available for *F. graminearum* revealed that for the distinction of a broad range of *F. graminearum* strains, no further microsatellites need to be developed. The available markers will be of valuable assistance in polyphasic studies addressing questions such as genotype associations in pathogenicity, toxigenicity, ecology, as well as species differentiation in the genus *Fusarium*. The data presented here demonstrates that the novel and the previously published microsatellite markers represent a powerful tool for differentiating strains of *F. graminearum*, the most prevalent FHB species in cereal samples.

Acknowledgements We thank the donors of *F. graminearum* strains and we are grateful to Irene Bänziger and Yvonne Häfele for excellent technical assistance.

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