Molecular differentiation and diagnosis of the cereal pathogens *Fusarium culmorum* and *F. graminearum*

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Randomly amplified polymorphic DNA (RAPD) markers were used to differentiate and identify cereal pathogenic *Fusarium* species and to study genetic variation in natural pathogen populations. In a survey of 71 *Fusarium* spp. isolates from different geographic regions, 25 selected decamer primers were used to amplify polymorphic fragments. Cluster analysis of band sharing coefficients separated isolates of *F. avenaceum*, *F. crookwellense*, *F. culmorum*, and *F. graminearum* into distinct species groups. Intraspecific variability was significantly higher in *F. graminearum* than in *F. culmorum* and the other species. Isolates of *F. graminearum* split into two main clusters corresponding to Groups 1 and 2. Isolates of *F. crookwellense* grouped between those of *F. culmorum* and *F. graminearum*, corroborating their known close relationship to both species. No associations were observed comparing the host and/or geographical origin of isolates within a species to the clusters obtained from the RAPD data. Based on sequences of two differential RAPD fragments, species-specific PCR assays were developed for *F. culmorum* and *F. graminearum* Group 2. Similarly, a diagnostic test was generated for *F. avenaceum* utilizing sequence polymorphism of the internal transcribed spacer regions of the nuclear ribosomal DNA. Screening a broad range of isolates of *Fusarium* spp., other cereal pathogens, and host plant DNA revealed no cross reactions in any of the three assays.

Keywords: *Fusarium* spp., cereal diseases, arbitrarily primers, genetic variation, diagnosis.

Fusarium culmorum (W.G. Smith) Sacc. and *F. graminearum* Schwabe are widespread fungal pathogens causing serious foot rot and head blight diseases of small grain cereals, grasses, and ear and stalk rot of corn. Severe infections result in tremendous yield and quality losses (Cook, 1981). Frequently, mixed infections of *Fusarium* spp. and other foot and root rotting pathogens, such as *Pseudocercosporella herpotrichoides* (Fron) Deighton and *Microdochium nivale* (Fries) Samuels & Hallett are observed on lesions of the same plant. As a consequence, identifying the pathogens on stem base symptoms is often difficult. Due to a lack of effective fungicides, crop resistance in cereals have become very important and suitable sources

of resistance are being explored intensively. To develop efficient breeding strategies, information about the genetic variability in natural pathogen populations appears crucial. Common methods for identifying *Fusarium* species employ cultural characteristics, however, phenotypic variation is abundant and traits are highly dependent on environmental conditions (Puhalla, 1981). In addition, morphological characters exhibit variation on a continuous scale with overlaps between the closely related species *F. culmorum* and *F. graminearum*. This situation is further complicated by the recognition of a separate species, *F. crookwellense* Burgess, Nelson & Toussoun that shares characteristics of *F. culmorum* and *F. graminearum* (Burgess & al., 1982). Hence, molecular markers, such as randomly amplified polymorphic DNA (RAPD; Williams & al., 1990) and other markers based on the polymerase chain reaction (PCR) offer very promising alternatives as versatile and informative molecular tools.

The objectives of our investigations were (1) to assess RAPD markers suitable for differentiation and identification of the closely related *Fusarium* species, (2) to study genetic variation within and between the species and in natural pathogen populations, and (3) to develop reliable and sensitive diagnostic assays for species-specific detection of the pathogens *F. culmorum* and *F. graminearum* on their cereal hosts.

Materials and methods

Fungal cultures

A total of 73 single-spored isolates of *F. crookwellense*, *F. culmorum*, *F. graminearum* Groups 1 and 2, and for comparison *F. avenaceum*, *Microdochium nivale* var. *nivale* and *Pseudocercosporella herpotrichoides* var. *acuformis* were examined (Tab. 1).

Tab. 1. – Isolates of *Fusarium* spp., *Microdochium nivale* and *Pseudocercosporella herpotrichoides* surveyed with RAPD markers.

Species	Isolate code	Original host/ habitat	Geographical origin	Source*	Original code
F. avenaceum	Fa1.1	*Dianthus* sp.	Great Britain	1	CBS121.73
	Fa1.2	*Dianthus* sp.	Germany	2	DSM62161
	Fa3	*Secale cereale*	Germany	3	#§
	Fa4	*S. cereale*	Germany	3	#
F. crookwellense	Fckw1	-	Germany	4	63558
	Fckw2	-	Germany	4	64483
	Fckw3	-	Germany	4	64545

Tab. 1. – continued

Species	Isolate code	Original host/ habitat	Geographical origin	Source*	Original code
F. culmorum	Fc1	*Triticum aestivum*	Germany	4	62191
	Fc2	*Hordeum vulgare*	Finland	4	64218
	Fc3	Soil	Norway	4	64223
	Fc3.1	*T. aestivum*	-	1	CBS251.52
	Fc3.2	*T. aestivum*	Great Britain	1	CBS122.73
	Fc3.3	*S. cereale*	-	1	CBS250.52
	Fc3.4	*Zea mays*	Germany	2	DSM62184
	Fc3.7	-	Canada	10	HLX1503
	Fc3.8	*T. aestivum*	Bulgaria	2	DSM62223
	Fc3.9	-	Germany	3	FG13
	Fc3.10	*Vicia faba*	Great Britain	7	26-2
	Fc7	*T. aestivum*	Great Britain	11	RPB5161
	Fc9	*S. cereale*	Germany	3	#
	Fc11	*T. aestivum*	Germany	3	#
	Fc15	*T. aestivum*	Germany	3	#
	Fc16	*S. cereale*	Germany	3	#
	Fc19	*T. durum*	Germany	3	#
	Fc22	*S. cereale*	Germany	3	#
	Fc27	*S. cereale*	Germany	3	#
	Fc29	*T. aestivum*	Germany	3	#
	Fc30	*T. dicoccum*	Germany	3	#
	Fc34	*S. cereale*	Poland	3	#
	Fc36	*S. cereale*	Germany	3	#§
	Fc40	*T. aestivum*	Hungary	6	-
	Fc46	*T. aestivum*	The Netherlands	8	39-01
	Fc55	*T. aestivum* ssp.	Germany	3	#
	Fc66	*T. aestivum*	Italy	12	ITEM120
	Fc68	*T. aestivum*	The Netherlands	8	SVP8901
	Fc70	*T. aestivum*	Switzerland	8	SVP8906
	Fc71	*Z. mays*	Australia	5	F4007
	Fc74	-	USA	9	-
	Fc436	-	Finland	8	436
F. graminearum[II]	Fg2	-	-	3	#
	Fg3	*T. durum*	-	4	-
	Fg7	*T. aestivum*	Yugoslavia	3	#
	Fg7.1	*T. aestivum*	The Netherlands	1	CBS389.62
	Fg7.2	*Musa* sp.	Honduras	1	CBS415.86
	Fg7.3	*Z. mays*	South Africa	1	CBS316.73
	Fg7.5	*Z. mays*	Germany	2	DSM4528
	Fg7.6	*Z. mays*	USA	2	DSM1095
	Fg7.7	*Lolium* sp.	Germany	2	DSM62722
	Fg7.8	*Avena sativa*	Germany	2	DSM62050
	Fg7.9	*Z. mays*	Germany	2	DSM4527
	Fg7.10	*T. aestivum*	Great Britain	7	F19
Group 1	Fg7.11	*T. aestivum*	Australia	5	F5647
Group 1	Fg7.12	*T. aestivum*	Australia	5	F10876
Group 1	Fg7.13	*T. aestivum*	Australia	5	F4253
Group 1	Fg7.14	*T. aestivum*	Australia	5	F11132
Group 1	Fg7.15	*T. aestivum*	Australia	5	F11133

Tab. 1. – continued

Species	Isolate code	Original host/ habitat	Geographical origin	Source*	Original code
Group 2	Fg7.16	*Lolium* sp.	Australia	5	F6894
F. graminearum[II]					
Group 2	Fg7.17	*Z. mays*	Australia	5	F1402
Group 2	Fg7.18	*H. vulgare*	Australia	5	F1625
Group 2	Fg7.19	*Triticale*	Australia	5	F6893
Group 2	Fg7.20	*Paspalum* sp.	Australia	5	F1819
	Fg8	*S. cereale*	Germany	3	#[§]
	Fg10	*S. cereale*	Germany	3	#
	Fg11	*T. aestivum*	Germany	3	#
	Fg12	*S. cereale*	Germany	3	#
	Fg22	*T. aestivum*	Hungary	6	-
	Fg23	*S. cereale*	Hungary	6	-
	Fg25	*S. cereale*	Italy	3	#
	Fg26	-	USA	9	-
	Fg27	*H. vulgare*	Germany	3	#
	Fg28	*Z. mays*	Poland	3	#
M. nivale var. *nivale*					
	Mn4	*S. cereale*	Germany	3	GN4/3
P. herpotrichoides var. *acuformis*	Pha9	*S. cereale*	Germany	13	C39A

* 1 = Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; 2 = Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; 3 = Th. Miedaner, Stuttgart, Germany; 4 = H. Nirenberg, Berlin, Germany; 5 = L. W. Burgess, Sydney, Australia; 6 = A. Mesterhazy, Szeged, Hungary; 7 = P. Nicholson, Norwich, Great Britain; 8 = C. H. A. Snijders, Wageningen, The Netherlands; 9 = Department of Plant Pathology, St. Paul, USA; 10 = J. D. Miller, Ottawa, Canada; 11 = Rothamsted Exp. Station, Harpenden, Great Britain; 12 = M. Solfrizzo, Bari, Italy; 13 = H. Fehrmann, Göttingen, Germany.

[§] Same isolate code as given in the second column.

[II] Group assignment of these isolates of *F. graminearum* has not been determined or, where stated, it has been done by L. W. Burgess.

DNA extraction

Mycelium was grown from single-spored cultures and total genomic DNA was isolated according to the microextraction protocol of Möller & al. (1992). The DNA was quantified by UV spectrophotometry at 260 nm and by comparison to appropriate DNA standards using agarose gel electrophoresis.

RAPD-PCR

Based on the original RAPD technique of Williams & al. (1990) a standard protocol was developed for amplifying reproducible and scorable fragments of *Fusarium* spp. genomic DNA (Schilling & al., 1994a). Overall,

25 decamer primers of arbitrary base composition were analyzed which had been selected through a screening experiment from a total of 120 primers (kit UBC100/1, University of British Columbia, Vancouver, Canada and kit T of Operon Technologies, Alameda, California). A preliminary report of this research has been published (Schilling & al., 1994b).

Data analysis

Amplification products were visually examined and fragment sizes determined. Presence or absence of each fragment size class was scored as 1 and 0, respectively. The resulting matrix was used to compute Dice's similarity coefficient (Dice, 1945) for all possible pairwise comparisons of the isolates. Cluster analysis by the 'unweighted pair-group method of arithmetic averages' (UPGMA; Sneath & Sokal, 1973) were performed with the similarity values using the SAHN procedure of the program NTSYS-pc Version 1.7 (Rohlf, 1992).

Species-specific PCR

Detailed protocols for developing species-specific PCR assays for *F. culmorum* and *F. graminearum* that are based on sequence information of differential RAPD fragments are described elsewhere (Schilling & al., 1996).

Results

RAPD assessment and primer survey

In a screening experiment, 120 decamer primers of arbitrary base composition were surveyed for amplifying polymorphic fragments among six single-spored isolates each of *F. culmorum* and *F. graminearum* that had been selected from diverse geographic regions (Schilling & al., 1994a). Most primers (88) generated scorable fragments in the size range of 0.25 to 3.5 kbp that distinguished between the species. Profiles of 37 primers were highly polymorphic among *F. graminearum* isolates. In contrast, only 12 primers revealed variation among six *F. culmorum* isolates.

Genetic diversity within and between *Fusarium* species

Based on the results of our primer screening, 25 primers were selected that revealed the most polymorphic fragments within each

species. These were used to investigate the degree of genetic diversity among a collection of 71 isolates of *Fusarium* spp., and one isolate of *M. nivale* and *P. herpotrichoides* (Tab. 1) each. These isolates came from different geographic locations and continents and from diverse hosts. In Fig. 1, RAPD profiles of representative isolates of *Fusarium* spp., *M. nivale*, and *P. herpotrichoides* are presented that were amplified with primer UBC8. The fragments are polymorphic mainly at the species level. Thus, primer UBC8 provides RAPD markers that are suitable for species identification. In contrast, Fig. 2 shows highly polymorphic fragments of representative isolates of *F. culmorum* and *F. graminearum* that were amplified with primer UBC28. This primer reveals a high degree of variation among isolates within both species and is useful to distinguish individual isolates.

Among the 73 isolates, a total of 599 different fragment size classes were scored and used to calculate Dice's coefficients of genetic similarity for all possible pairwise comparisons. The arithmetic mean of genetic similarities amounted to 0.83 among isolates of *F. culmorum*, 0.78 among isolates of *F. crookwellense*, 0.63 among isolates of *F. avenaceum*, and 0.62 among isolates of *F. graminearum* with standard deviations of 0.03, 0.08, 0.06, and 0.23, respectively. Hence, intraspecific variation was significantly higher in *F. graminearum* than in *F. culmorum* and the other species. UPGMA cluster analysis (Fig. 3) separated the isolates of *F. avenaceum*, *F. crookwellense*, *F. culmorum* and *F. graminearum* into distinct species groups. Species-specific RAPD markers identified two putatively classified '*F. graminearum*' isolates (Fg10 and Fg27) that exhibited distinct patterns of *F. crookwellense* and *F. avenaceum*, respectively. Isolates of *P. herpotrichoides* and *M. nivale* were clearly distinct from *Fusarium* spp. with weak final linkages of less than 10% genetic similarity. Coefficients of similarity between *Fusarium* isolates ranged from 12% to 40% for interspecific and 24% to 96% for intraspecific comparisons. Isolates of *F. crookwellense* clustered between those of *F. culmorum* and *F. graminearum*, corroborating known taxonomic relationships among these three species. Isolates of *F. graminearum* split into two distinct classes corresponding to Groups 1 and 2. Among *F. graminearum* Group 2, further subclusters occurred indicating a complex population structure. In contrast, *F. culmorum* showed an undifferentiated variability pattern. No associations were observed comparing the RAPD-based groupings within *F. culmorum* and *F. graminearum* with the host and/or geographic origin of the isolates. Similarly, marker-based associations among *F. culmorum* isolates did not coincide with the degree of aggressiveness as determined in a field experiment by Miedaner & al. (1996).

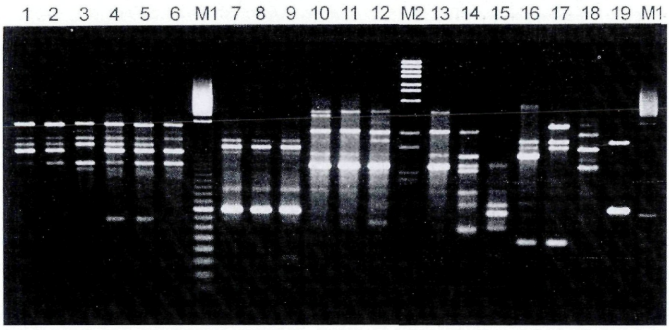

Fig. 1. – Species-specific RAPD patterns of different *Fusarium* spp., *Microdochium nivale* and *Pseudocercosporella herpotrichoides* amplified with arbitrary primer UBC8. Fragments were resolved in 1.5% agarose and stained with ethidium bromide. – Lanes 1 to 6: *F. culmorum*, lanes 7 to 9: *F. graminearum* Group 2, lanes 10 to 13: *F. graminearum* Group 1, lanes 14, 15: *F. avenaceum*, lanes 16, 17: *F. crookwellense*, lanes 18, 19: *M. nivale* and *P. herpotrichoides*, respectively. Lanes M1 and M2 are molecular weight standards (100-bp-ladder of Pharmacia Biotech and *Bst*EII digested Lambda DNA, respectively).

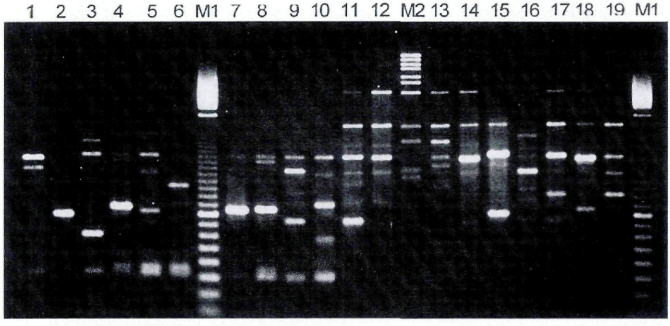

Fig. 2. – Agarose gel showing highly polymorphic RAPD fragments of 10 representative isolates of *F. culmorum* (lanes 1 to 10) and 9 isolates of *F. graminearum* Group 2 (lanes 11 to 19) amplified with arbitrary primer UBC28. Molecular weight standards M1 and M2 are as in Fig. 1.

Species-specific PCR assays

RAPD fragments were identified that differentiated between *F. culmorum* and *F. graminearum* Group 2. By cloning and sequencing two fragments, pairs of specific primers of each 20 nucleotides were synthesized. Single, easily distinguishable fragments of about 470 and 330 bp length were amplified with each of the primer pairs. Similarly, a PCR assay was developed for *F. avenaceum* utilizing sequence polymorphism of the internal transcribed spacer regions of the nuclear ribosomal DNA. A 270 bp fragment was exclusively amplified from genomic DNA of *F. avenaceum*. Screening 150 isolates of 14 different *Fusarium* species and isolates of 7 fungal genera that are common cereal pathogens revealed no cross reactions in any of the three assays. Furthermore, none of the primer pairs amplified host plant DNA, extracted from rye and maize. The assays proved reliable, robust, and sensitive in detecting fungal DNA of individual *Fusarium* spp. in raw extracts of infected host tissues such as stem bases and seeds of wheat and rye.

Discussion

In the presented study, RAPDs of *Fusarium* spp. proved to be a sensitive technique for characterizing genetic diversity between and within the species. Isolates of *F. crookwellense*, *F. culmorum* and *F. graminearum* could be easily differentiated by species-specific RAPD markers. Within *F. graminearum* two subgroups could be distinguished consisting of Group 1 and Group 2 isolates that represent differently adapted populations (Francis & Burgess, 1977). With respect to morphological characters, these two groups are hardly distinguishable. Differences, however, are noticed in the abundance of fertile perithecia that are produced on artificial medium. In the UPGMA clustering of RAPD data, the large genetic distance between isolates of Groups 1 and 2 is remarkable. In a comparative study by Benyon & al. (1995), restriction fragment length polymorphism (RFLP) was utilized for characterizing *Fusarium* species that are involved in crown rot diseases of cereals. In fact, their data are in good accordance with our results and support the distant relation between *F. graminearum* Groups 1 and 2. The authors suggest considering the two groups as different species (L. W. Burgess, pers. comm.).

Between isolates of *F. culmorum* and *F. graminearum* Group 2 marked differences in the variability patterns were observed. These differences could be attributed in part to the prevailing mode of

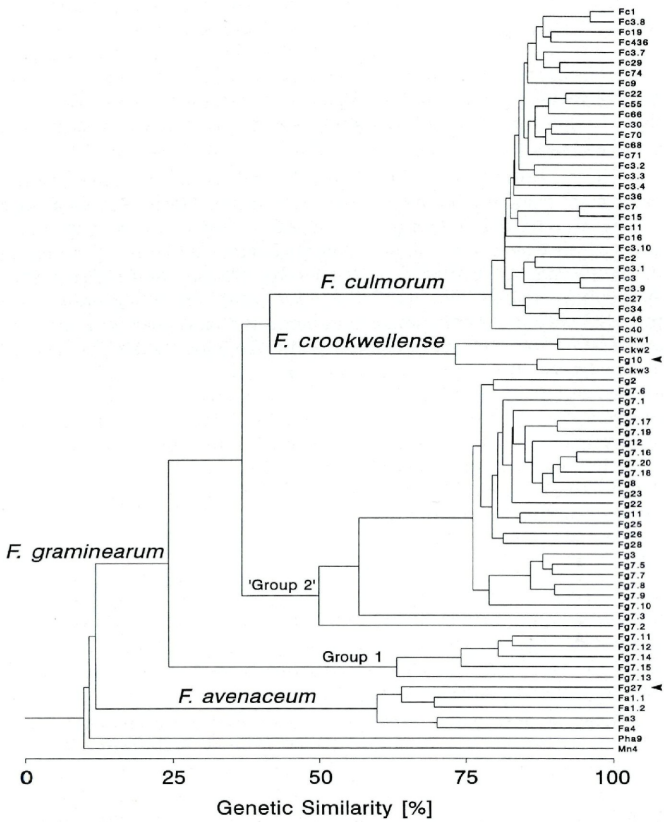

Fig. 3. – Cluster analysis (unweighted pair-group method, arithmetic averages) of RAPD data obtained for 71 *Fusarium* spp. isolates and one isolate each of *Microdochium nivale* and *Pseudocercosporella herpotrichoides*. Amplification products of 25 decamer primers yielded a total of 599 fragment size classes for calculating genetic similarities. Subclusters branching off at the species level are labeled with the respective taxon. Arrows pointing to two '*F. graminearum*' isolates (Fg10 and Fg27) were identified to belong to *F. crookwellense* and *F. avenaceum*, respectively.

reproduction by each species. *F. culmorum* propagates exclusively by conidia. In contrast, *F. graminearum* exhibits both reproductive modes. Besides conidia produced during the infection progress, the sexual stage *Gibberella zeae* (Schw.) Petch is usually formed once per year and ascospores are released at the beginning of the growing season (Sutton, 1982). Sexual recombination would result in increased genetic variation that could account for the broader range of diversity that we have observed in *F. graminearum* and it is likely to enable the species to adapt more easily to variable hosts and environments.

The majority of isolates of *F. graminearum* investigated originated from temperate regions of Europe, North America, and Australia and are thought to represent Group 2. Among these genotypes we detected a higher level of genetic variability than among *F. culmorum*. In contrast, a survey by Ouellet & Seifert (1993) employing RAPDs for assessing variability in *F. graminearum* revealed only little polymorphism among the examined isolates. The discrepancy of their results with ours are most likely caused by differences in the experimental set-up of the RAPD technique, e.g. concentration of template DNA, number of cycles of the PCR, and source of the *Taq* polymerase. Particularly, differences in the number of primers and isolates used in the screening for polymorphism have probably led to the contrasting results (Möller & al., 1994). Supporting our findings, Bowden & Leslie (1992) reported extensive variability within *F. graminearum* by investigating vegetative compatibility even among isolates obtained from a narrow geographic region.

We have started to analyze fungal populations consisting of isolates of *F. culmorum* and *F. graminearum* from naturally infected wheat and rye. Hierarchically sampled isolates are surveyed with informative primers that are useful to genotype individuals. This will allow us to study the genetic structure and diversity of natural *Fusarium* populations.

Species-specific RAPD markers were efficiently converted into differential PCR assays for the detection of *F. culmorum*, *F. graminearum*, and *F. avenaceum*, respectively. The assays are highly sensitive and provide reliable tools which could facilitate the diagnosis of *Fusarium* spp. and aid in screening breeding materials for *Fusarium* resistance.

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