J Phytopathol 157:446–456 (2009)
doi: 10.1111/j.1439-0434.2008.01525.x

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Genetic Variability of Phytopathogenic *Fusarium proliferatum* Associated with Crown Rot in *Asparagus officinalis*

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Received July 25, 2008; accepted October 5, 2008

Keywords: aminoacyl transferase, DAF-PCR, genetic fingerprinting, PCR–RFLP, polyketide synthase

Abstract

Fusarium proliferatum (teleomorph: *Gibberella intermedia*) is a causal agent of crown rot of *Asparagus officinalis* and is one potential fumonisin-producing species within the genus *Fusarium*. It colonizes roots and crowns of asparagus plants, but could also be isolated from symptomless asparagus spears. *Fusarium proliferatum* isolates obtained from perennial asparagus plantings from Austria and Germany were included in a study on detectability and variability of two essential genes of the fumonisin-gene cluster. Genetic fingerprinting of 45 isolates revealed 14 different fingerprint groups, indicating genetic heterogenicity of *F. proliferatum*. Most isolates differentiated into three main fingerprint clusters, but no association was found between fingerprint group and origin of the isolates. By gene-specific PCR it was shown that, in 25 isolates tested, both initial genes of the fumonisin biosynthetic pathway – *FUM1*, encoding a polyketide synthase and *FUM8*, a gene for a putative aminoacyl transferase – were detectable. This suggests that these isolates were able to produce fumonisins and could contribute to the detected contamination in originating asparagus spears with this mycotoxin. Thus, early detection of *FUM*-genes in *F. proliferatum*-colonized asparagus may be suited to prevent uptake of fumonisin contaminated food with the human diet. Restriction fragment length polymorphism analysis (PCR-RFLP) of the amplified *FUM* gene fragments revealed little sequence variability, suggesting a conserved structure of these genes within this species. However, sequence analysis confirmed intraspecific nucleotide polymorphisms of these genes.

Introduction

Fusarium proliferatum (*F. proliferatum*, teleomorph: *Gibberella intermedia*) is one of the main causal agents of crown and root rot, a destructive disease of asparagus (*Asparagus officinalis*). Infection of plants with *F. proliferatum* or other *Fusarium* spp. often leads to asparagus decline, and a decrease in yield to unprofitable levels in plantings that have been established for 8–10 years. Accumulation of soil-borne fusarial pathogens is thought to result in depressed yields when affected fields are replanted with asparagus (Blok and Bollen, 1996); such fields sometimes never produce profitable amounts of asparagus spears. The species composition of asparagus crown and root rot varies according to climatic conditions, *F. proliferatum* being the predominant species in warmer regions (Doohan et al., 2003). However, Vujanovic et al. (2006) report that this species was ubiquitous in asparagus fields across five different ecological areas in North America, including areas with climates similar to northern Europe. Therefore, *F. proliferatum* may be found more frequently in Germany in the near future.

Fusarium proliferatum is a well known producer of polyketide derived B type fumonisins. Accumulation of high levels of these secondary metabolites has been described for several *F. proliferatum* strains (Rheeder et al., 2002; Desjardins, 2006), including isolates infecting asparagus spears (Logrieco et al., 1998; Seefelder et al., 2002; Weber et al., 2006). Fumonisin is synthesized and production is regulated by proteins encoded by 15 genes organized as a gene cluster (Seo et al., 2001; Proctor et al., 2002, 2003). The fumonisin biosynthetic gene cluster is thought to occur only in fumonisin producing strains of *Fusarium* fungi (Waalwijk et al., 2004; Desjardins, 2006). A synthetic pathway has been proposed by Bojja et al. (2004). Key enzymes catalyzing the first steps are a polyketide synthase (*FUM1*) and an aminoacyl transferase (*FUM8*), whose disruption leads to elimination of fumonisin production in *Fusarium verticillioides* (Proctor et al., 1999; Bojja et al., 2004).

Major efforts have been made within the European Union to minimize uptake of mycotoxins with the human diet. Contamination of asparagus spears with fumonisins has been reported previously (Seefelder et al.,

2002; Gossmann et al., 2005). Thus, the colonization with and differentiation of fumonisin producing fusaria in harvested asparagus spears should be closely monitored. This can be facilitated by application of molecular methods, which are suitable for sensitive pathogen detection especially at early stages of infection, before disease symptoms become visible. Intron-rich genes encoding e.g. calmodulin (Mule et al., 2004) β-tubulin (Seifert and Levesque, 2004) and the α-subunit of translation elongation factor 1 (Geiser et al., 2004) are often used as molecular markers for species discrimination and sequence-based phylogenies within the genus *Fusarium*. By specific PCR amplification of fumonisin-biosynthesis genes – as suggested for instance by Konietzny and Greiner (2003) for detection of mycotoxigenic fungi in foods – cross reactivity of primers with the plant genome or other associated microorganisms can be avoided. In addition to a general detection of fumonisin-producing fusaria, species identification might be possible. To attain both goals simultaneously, putative target genes have to have a certain degree of conservation within a species, but should exhibit adequate interspecific sequence diversity.

Our objectives were to determine DNA polymorphisms among *F. proliferatum* that colonize asparagus spears. Concomitantly, the presence of two candidate genes from the fumonisin biosynthetic pathway was probed in *F. proliferatum* isolates, originating mainly from fumonisin contaminated asparagus spears. Sequence variability of these genes were analyzed and related to the whole-genome genetic variability using fingerprint methods.

Materials and Methods

Sampling and maintenance of *Fusarium proliferatum* isolates

Fifty-seven isolates were originally obtained from asparagus spears (22–35 cm in length) harvested from perennial plantings in Austria and Germany in 2002–2004 (Table 1). Asparagus stands with nine different cultivars were established between the years 1993 and 1998. Samples were collected in May and June during the main harvest period. Fungal cultures were established on SNA (Spezieller Nährstoffarmer Agar, Nirenberg, 1976) as described in Gossmann et al. (2005) for determination of *Fusarium* species according to morphological characteristics (Booth, 1971; Nirenberg, 1976; Gerlach and Nirenberg, 1982). Isolates were kept in soil cultures at 8°C within the culture collection of the Section Phytomedicine of the Humboldt-Universität zu Berlin. *Fusarium proliferatum* isolates were activated from stocks on malt extract agar (20 g malt extract/l) and transferred onto SNA after 4 days. After microscopical reassessment of fungal species, *F. proliferatum* isolates were grown for 7 days in 10 ml potato dextrose medium (PDM) on a rotary shaker at room temperature for DNA isolation.

DNA extraction

Mycelium from PDM cultures was collected by vacuum filtration on nitrocellulose membranes (pore size 0.6 μm; Sartorius, Germany), washed with 5 ml of 10 mM Tris–Cl, pH 8.0, 1 mM EDTA (TE buffer) and DNA was isolated by an upscaled protocol described by Cenis (1992). Briefly, mycelia and filters were ground in liquid N_2 and incubated for 2 h on a rotary shaker in tubes containing 900 μl extraction buffer (200 mM Tris–Cl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). After the addition of 450 μl 3 M sodium acetate, pH 5.2, tubes were incubated at −20°C for 10 min. After thawing, tubes were centrifuged at 11 000 ***g*** for 5 min and supernatants were mixed with equal volumes of 2-propanol. After 5 min at room temperature, nucleic acids were precipitated by centrifugation (15 000 × ***g***, 4°C, 10 min) and washed once with 1 ml 70% ethanol. DNA pellet was dried and dissolved in 50 μl TE buffer.

Genetic fingerprinting of *Fusarium proliferatum* DNA

Random amplified polymorphic DNA-PCR (RAPD-PCR; Williams et al. (1991) and DNA amplification fingerprinting (DAF-PCR, Caetano-Anolles et al., 1991) were done with 25 ng of fungal DNA in 50 μl PCR reactions. The reaction mixture further consisted of 10 mM Tris–Cl, pH 9.0, 50 mM KCl, 0.1% (w/v) Triton, 1.5 mM $MgCl_2$, 200 μM of each dNTP and 2.5 U of *Taq*-Polymerase (Fermentas, St Leon-Rot, Germany). For RAPD-PCR, 1.25 μM of a short decamer oligonucleotide, either Q2 or Q6, (Roth, Karlsruhe, Germany) was added. DAF-PCR was carried out with two decamer primers in the reaction mixture with a final concentration of 5 μM each, applying the combinations Q2 + Q6, Q2 + Q20 or Q11 + Q20 (Roth). Cycling conditions for both fingerprint methods, carried out in a Robocycler (Gradient96; Stratagene, La Jolla, CA, USA), were 5 min at 95°C, 35 cycles with 30 s at 95°C, 30 s at 40°C, 30 s at 72°C, with a final extension for 7 min at 72°C. Amplified products were visualized under UV-light after separation in 1% agarose gels and ethidiumbromide staining. *Fusarium proliferatum* isolates were grouped according to discriminating fragments appearing in the RAPD- and DAF-PCR patterns.

Amplification of *FUM1*- and *FUM8*- genes and PCR-RFLP

Gene specific oligonucleotides were designed based on *F. proliferatum* (strain M-6993) sequences available at the NCBI database for the partial polyketide synthase gene, (*FUM1*, acc. AY577458) and oxamine-synthase like gene (*FUM8*, acc. AY577451) respectively. Primer-sequences were: Fpro-fum1-for 5′GAGCAATATAGGCTGTTACG, Fpro-fum1-rev 5′TCCATCCGAATTTGAAGATGT, amplifying 525 bp of *FUM1* and Fpro-fum8-for 5′TCTCCTGTTGTCTGCTTTCCA, Fpro-fum8-rev 5′GTAGTGAGAGCATCATAGTATG, producing an 801-bp product in PCR, applying 1 μl of fungal DNA preparation in a 50 μl reaction with 500 nM of each primer and 3.5 mM $MgCl_2$. Other PCR-reaction contents were identical to those described above. The PCR program for the amplification of *FUM1* fragments, conducted in a Tgradient

Table 1
Origin of fungal isolates, genetic fingerprint analysis and detection of fumonisin genes in *Fusarium proliferatum*

Fungal isolale	Origin	Asparagus cultivar	Fingerprint group	*FUM1* gene fragment [bp]	*FUM8* gene fragment [bp]	Fumonisin B1 concentration in host plant material[a] [μg/kg]	Fumonisin B1 concentration in vitro[b] [μg/kg corn-substrate]
Fusarium proliferatum obtained from asparagus spears							
Fpro-193	Brandenburg, Germany, June 2002	RAVEL	nd[c]	525[d]	801	< detection limit	165.4
Fpro-194	Brandenburg, Germany, June2002	EPOSS	nd	525	801[d]	< detection limit	170.4
Fpro-196	Brandenburg, Germany, June 2002	RAMOS	nd	525[d]	801[d]	< detection limit	181.4
Fpro-199	Brandenburg, Germany, June 2002	BACKLM	nd	525[d]	801	< detection limit	169.4
Fpro-202	Brandenburg, Germany, June 2002	THIELIM	nd	525[d]	801[d, e]	< detection limit	119.2
Fpro-204	Brandenburg, Germany, June 2002	GROLIM	nd	525[d]	801[d]	< detection limit	124.4
Fpro-224	Lower Austria I, Austria, May 2003	BOONLIM	G	nd	nd	14, spears	nd
Fpro-225	Lower Austria I, Austria, May 2003	BOONLIM	H	nd	nd	14, spears	nd
Fpro-226	Lower Austria I, Austria, May 2003	BOONLIM	A	nd	nd	17, spears	nd
Fpro-227	Lower Austria I, Austria, May 2003	BOONLIM	A	525 [d, e]	801[e]	23, spears; 320, roots + crown	nd
Fpro-228	Lower Austria I, Austria, May 2003	BOONLIM	B	nd	nd	17, spears	nd
Fpro-229	Lower Austria I, Austria, May 2003	BOONLIM	C	525	801	15, spears	nd
Fpro-230	Lower Austria I, Austria, May 2003	BOONLIM	A	525[d, e]	801[d]	11, spears	nd
Fpro-231	Lower Austria II, Austria, May 2003	BOONLIM	B	nd	nd	9, spears	nd
Fpro-232	Lower Austria II, Austria, May 2003	BOONLIM	B	nd	nd	9, spears	nd
Fpro-233	Lower Austria II, Austria, May 2003	BOONLIM	K	nd	nd	16, spears	nd
Fpro-234	Lower Austria II, Austria, May 2003	BOONLIM	L	525	801	16, spears	nd
Fpro-235	Lower Austria II, Austria, May 2003	BOONLIM	C	nd	nd	11, spears	nd
Fpro-236	Lower Austria II, Austria, May 2003	BOONLIM	C	nd	nd	11, spears	nd
Fpro-237	Lower Austria II, Austria, May 2003	BOONLIM	B	nd	nd	12, spears	nd
Fpro-238	Lower Austria II, Austria, May 2003	BOONLIM	E	nd	nd	12, spears	nd
Fpro-239	Lower Austria II, Austria, May 2003	BOONLIM	A	nd	nd	12, spears	nd
Fpro-240	Burgenland I, Austria, May 2003	SPARGANIVA	D	525	801	nd	nd
Fpro-241	Upper Austria, Austria, May 2003	GIJNLIM, BACKLIM	C	525 [d, e]	801 [d, e]	13 spears; 468 roots + crown	nd
Fpro-242	Upper Austria, Austria, May 2003	GIJNLIM, BACKLIM	C	nd	nd	13, spears	nd
Fpro-243	Upper Austria, Austria, May 2003	GIJNLIM, BACKLIM	C	nd	nd	10, spears	nd
Fpro-244	Upper Austria, Austria, May 2003	GIJNLIM, BACKLIM	A	525[d, e]	801[d]	12, spears	nd
Fpro-245	Burgenland II, Austria, May 2003	SPARGANIVA	E	525[d, e]	801	11, spears	nd
Fpro-246	Lower Austria III, Austria, May 2003	GIJNLIM	F	525	801	10, spears	nd
Fpro-247	Lower Austria I, Austria, June 2003	BOONLIM	E	525	801	48, spears	nd
Fpro-248	Lower Austria I, Austria, June 2003	BOONLIM	F	nd	nd	18, spears	nd
Fpro-249	Lower Austria I, Austria, June 2003	BOONLIM	M	nd	nd	17, spears	nd
Fpro-250	Lower Austria I, Austria, June 2003	BOONLIM	D	nd	nd	7, spears	nd

Table 1
(Continued)

Fungal isolale	Origin	Asparagus cultivar	Fingerprint group	*FUM1* gene fragment [bp]	*FUM8* gene fragment [bp]	Fumonisin B1 concentration in host plant material[a] [μg/kg]	Fumonisin B1 concentration in vitro[b] [μg/kg corn-substrate]
Fpro-251	Lower Austria I, Austria, June 2003	BOONLIM	A	nd	nd	12, spears	nd
Fpro-252	Lower Austria I, Austria, June 2003	BOONLIM	B	nd	nd	8, spears	nd
Fpro-253	Lower Austria I, Austria, June 2003	BOONLIM	C	nd	nd	8, spears	nd
Fpro-254	Lower Austria I, Austria, June 2003	BOONLIM	G	nd	nd	16, spears	nd
Fpro-255	Lower Austria I, Austria, June 2003	BOONLIM	A	nd	nd	16, spears	nd
Fpro-256	Lower Austria I, Austria, June 2003	BOONLIM	A	nd	nd	9, spears	nd
Fpro-257	Lower Austria I, Austria, June 2003	BOONLIM	B	nd	nd	10, spears	nd
Fpro-258	Lower Austria II, Austria, June 2003	BOONLIM	N	525	801	19, spears	nd
Fpro-259	Lower Austria II, Austria, June 2003	BOONLIM	O	nd	nd	21, spears	nd
Fpro-260	Lower Austria II, Austria, June 2003	BOONLIM	C	nd	nd	11, spears	nd
Fpro-261	Lower Austria III, Austria, June 2003	GIJNLIM	H	nd	nd	13, spears	nd
Fpro-262	Lower Austria III, Austria, June 2003	GIJNLIM	A	nd	nd	nd	nd
Fpro-263	Burgenland II, Austria, June 2003	SPARGANIVA	B	525	801	25, spears	nd
Fpro-264	Burgenland II, Austria, June 2003	SPARGANIVA	B	nd	nd	34, spears	nd
Fpro-265	Burgenland II, Austria, June 2003	SPARGANIVA	A	525	801	25, spears	nd
Fpro-266	Upper Austria, Austria, June 2003	GIJNLIM, BACKLIM	D	nd	nd	nd	nd
Fpro-267	Upper Austria, Austria, June 2003	GIJNLIM, BACKLIM	I	nd	nd	nd	nd
Fpro-268	Upper Austira, Austria, Juni 2003	GIJNLIM, BACKLIM	I	nd	nd	nd	nd
Fpro-269	Lower Austria I, Austria, Mai 2004	BOONLIM	nd	525	801	31, spears	nd
Fpro-271	Lower Austria I, Austria, May 2004	BOONLIM	nd	525[d, e]	801[d, e]	14, spears	nd
Fpro-277	Lower Austria II, Austria, May 2004	BOONLIM	nd	525[d, e]	801[d, e]	< detection limit	nd
Fpro-278	Lower Austria II, Austria, May 2004	BOONLIM	nd	525[d]	801[d, e]	< detection limit	nd
Fpro-279	Upper Austria, Austria, May 2004	GIJNLIM, BACKLIM	nd	525[d, e]	801[d, e]	11, spears	nd
Fpro-281	Lower Austria III, Austria, May 2004	GIJNLIM	nd	525[d]	801[d, e]	23, spears	nd

Note: Fumonisin B levels based upon dry weight were originally published in ([a]) Gossmann et al. 2007 and ([b]) Weber et al. 2006.
[c]not determined.
[d]determination of PCR-RFLP pattern.
[e]sequence obtained.

thermocycler (Biometra, Göttingen, Germany), was as follows: 2 min at 94°C, 40 cycles with 30 s at 94°C, 30 s at 53°C, 45 s at 72°C, with a final extension for 5 min at 72°C. *FUM8* fragments were amplified using an analogous program with an annealing temperature of 50°C and an extension time of 60 s. For PCR-RFLP analyses of *FUM1* and *FUM8* gene fragments, 10 μl of PCR product was digested with restriction enzymes *Alu*I, *Bsp*143I, *Bam*HI or *Rsa*I (Fermentas, St Leon-Roth, Germany). Restriction endonucleases were chosen, according to *in silico* digestion of corresponding gene fragments from public databases and were suitable to distinguish the *F. proliferatum* sequences from other fumonisin-producing species, such as *F. verticillioides* (*FUM1*, acc. AY577458, *FUM8*, acc. AF155773), *Fusarium oxysporum* (*FUM1*, acc. AY577457, *FUM8*, acc. AY577450), *Fusarium globosum* (*FUM1*, acc. AY577455, *FUM8*, acc. AY577448) and *Fusarium anthophilum* (*FUM1*, acc. AY577453, *FUM8*, acc. AY577446). After electrophoresis in a 2%

agarose gel, restriction patterns were visualized as described above.

Cloning and sequence analysis

PCR products of amplified *FUM* gene fragments from *F. proliferatum* isolates were ligated into pGEMTeasy (Promega, Madison, WI, USA) and transformed into chemocompetent *Escherichia coli*, using standard protocols (Sambrook et al., 1989). Plasmids were purified and sequenced from both directions using a BigDye® Terminator v1.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) after standard protocol and an ABI PRISM® 310 Genetic Analyzer from Applied Biosystems. Sequences were assembled in the freeware tool BioEdit 7.4 developed by Hall (1999) and sequence comparisons were conducted by CLUSTALW (Thompson et al., 1994). Phylogenetic reconstructions were done with the PHYLIP package (Felsenstein, 1989) applying maximum parsimony-, maximum likelihood- and neighbour joining-algorithms. Generated trees were compared and bootstrap analysis (Efron, 1979) with 1000 trials was performed using the maximum parsimony data sets.

Results

Fusarium proliferatum strains included in the study were either isolated from fumonisin B1 (FB1) contaminated spears or were isolates capable of producing FB1 *in vitro* (Table 1). To determine genetic variability among *F. proliferatum* genomes, genetic fingerprinting by RAPD and DAF-PCR was carried out by testing 45 *F. proliferatum* isolates originating from plantings in five locations in Austria. RAPD and DAF analysis, as shown in Fig. 1, discriminated 14 different patterns, leading to fingerprint groups A to O, but three fingerprint groups contained more than 50% of all investigated isolates (Table 1). Ten isolates produced identical patterns and are clustered within group A, while eight isolates each constituted groups B and C respectively. Remaining isolates were composed as clusters of three (fingerprint groups D, E) or two (fingerprint groups F to I) and isolates with individual fingerprint patterns (K to O). Some *F. proliferatum* isolates within a fingerprint cluster originated from the same location and even same asparagus spear, e.g. Fpro-231 and Fpro-232 (Fingerprint group B), Fpro-235 and Fpro-236 (group C) as well as Fpro-241 and Fpro-242 (group C). However, fingerprint groups could not be associated with geographic origin, asparagus cultivar or time of sampling. Interestingly, isolates obtained from the same asparagus spears, such as Fpro-238 and Fpro-239 or Fpro-252 and Fpro-253, were assigned to different fingerprint clusters. Moreover, fingerprint group A contained six of 18 isolates obtained from a plantation in lower Austria (location I), three and two isolates respectively belonged to fingerprint clusters B and C and one or two isolates from this location were also present in groups D to H and M.

The presence of the two initial genes of the fumonisin gene cluster (*FUM1* and *FUM8*) was checked in 13

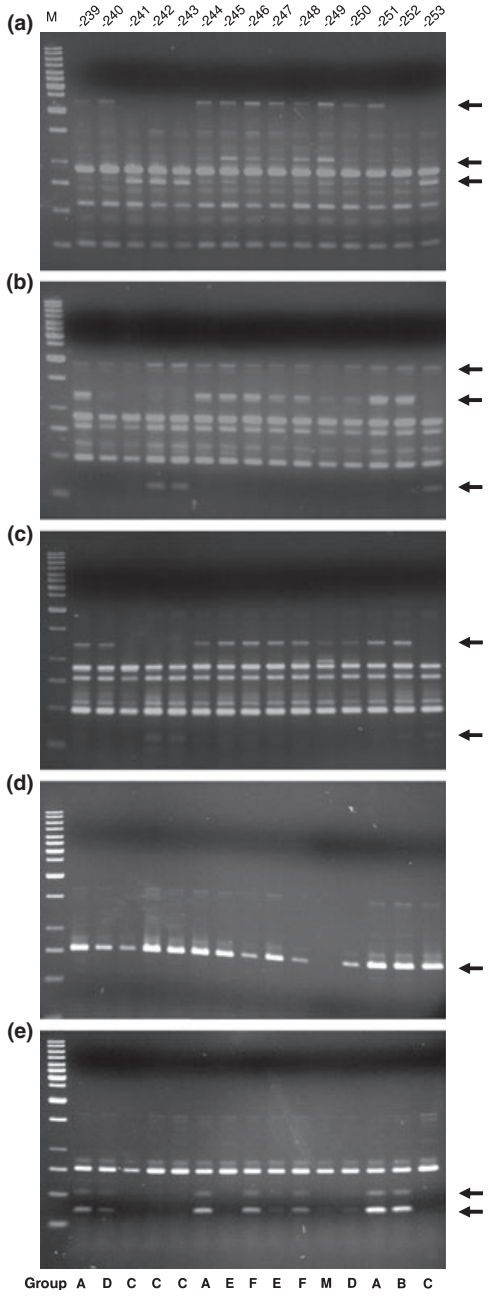

Fig. 1 Genetic fingerprinting of *Fusarium proliferatum* isolates applying RAPD-PCR with Q1 (a) and Q6 (b) primers and DAF-PCR with primer combinations Q2, Q6 (c) Q2, Q20 (d) and Q11, Q20 (e). Fragments of diagnostic value are indicated by an arrow. M = marker 1-kbp ladder, Fermentas

F. proliferatum isolates, which were characterized by RAPD and DAF-PCR and clustered within different fingerprint groups. Additionally, 12 fungal isolates originating from asparagus spears sampled in 2004 in Austrian stands and plantings in Brandenburg, Germany were subjected to *FUM*-gene specific PCR. Both genes were detectable in all 25 isolates analyzed (Table 1). *FUM* genes were detected in six *F. proliferatum* strains isolated from spears, without a traceable fumonisin B contamination. However, these strains were capable of FB1 production *in vitro*.

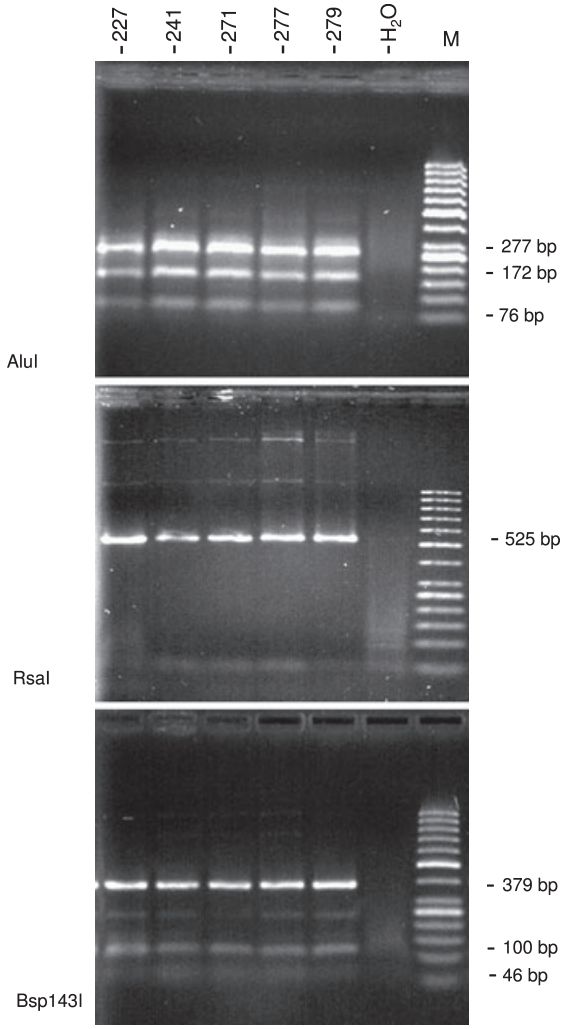

Fig. 2 PCR-RFLP pattern after restriction of *FUM1* fragments, amplified from *Fusarium proliferatum* isolates originating from asparagus, with *Alu*I, *Rsa*I and *Bsp*143I. Strain characteristics are outlined in Table 1. M = marker 50-bp ladder, Fermentas. Fragment sizes are indicated on the right omitting fragments smaller than 50 bp

Restriction fragment length polymorphism analyses of PCR amplified 525-bp *FUM1* fragments from 17 isolates revealed identical restriction patterns after digestion with *Alu*I, *Bsp*143I and *Rsa*I. The RFLP patterns generated (examples given in Fig. 2) were as expected from *in silico* digestion of the corresponding *FUM1 F. proliferatum* sequence received from the database (acc. AY577458). Generally, fragments generated after digestion of thirteen 801-bp *FUM8* fragments with restriction endoucleases *Alu*I, *Bsp*143I, *Rsa*I and *Bam*HI produced the expected fragment sizes, as calculated from the *FUM8* reference sequence (*F. proliferatum*, acc. AY577451). In a single case *Alu*I digestion of the *FUM8* fragment amplified from isolate Fpro-227 revealed an additional restriction site originating in the digestion of the 124-bp fragment into 73- and 51-bp fragments (Fig. 3). However, the obtained RFLP-pattern was unique and did not interfere with patterns from other *Fusarium* spp. calculated from the sequences available in the database (data not shown).

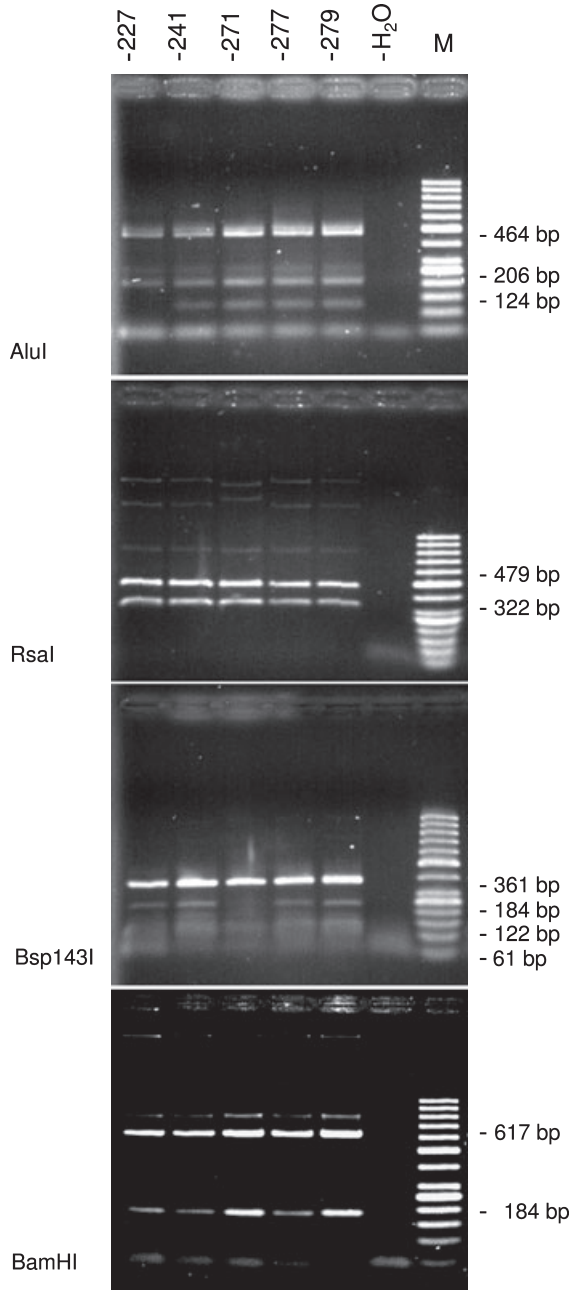

Fig. 3 PCR-RFLP pattern after restriction of *FUM8* fragments, amplified from *Fusarium proliferatum* isolates originating from asparagus, with *Alu*I, *Rsa*I, *Bsp*143I and *Bam*HI. Strain characteristics are outlined in Table 1. M = marker 50-bp ladder, Fermentas. Fragment sizes are indicated on the right omitting fragments smaller than 50 bp

Sequences of the *FUM1* and *FUM8 F. proliferatum* fragments were obtained after cloning of PCR-products. *FUM1* gene fragments from eight different isolates uniformly exhibited a 525-bp length, including primer sequences. Similarly, *FUM8* PCR-products amplified from eight *F. proliferatum* strains were 801-bp long; this included isolates which grouped into separate fingerprint clusters (Table 2). Thus, corresponding restriction patterns of the *FUM1* and *FUM8* sequence fragments derived from *F. proliferatum* were obtained in both the *in silico* and *in vitro* analyses.

Table 2
Nucleotide sequence identity in percent [%] by pairwise comparison of *FUM1*- and *FUM8*-gene fragments of *Fusarium* spp.

	FUM1					FUM8				
	n[a]	[bp][b]	Mean (S.D.)	Min	Max.	n[a]	[bp][b]	Mean (S.D.)	Min	Max.
Intraspecific sequence identity										
F. proliferatum	11	484	99.59 (± 0.25)	99.1	ID[c]	10	758	99.54 (± 0.21)	99.0	ID
F. verticillioides	10	488	99.70 (± 0.27)	99.1	ID	2	775	ID	ID	ID
Interspecific sequence identity of *F. proliferatum* isolates										
Gibberella fujikuroi species complex										
F. fujikuroi	1	484	96.91 (± 0.22)	96.6	97.1	1	758	98.63 (± 0.18)	98.4	99.0
F. globosum	1	484	96.24 (± 0.17)	96.0	96.4	1	758	98.50 (± 0.18)	98.2	98.9
F. antophilum	1	484	92.94 (± 0.17)	92.7	93.1	1	759	90.53 (± 0.19)	90.3	90.9
F. nygamai	1	489	84.84 (± 0.17)	84.6	85.0	1	783	76.92 (± 0.21)	76.7	77.4
F. verticillioides	10	488	84.32 (± 0.25)	83.6	84.6	2	775	77.17 (± 0.17)	77.0	77.6
Fusarium oxysporum species complex										
F. oxysporum	1	484	86.24 (± 0.17)	86.0	86.4	1	757	81.19 (± 0.20)	81.0	81.6

[a]sequences per Fusarium species included in the pairwise comparison.
[b]compared fragment length without primer sequences.
[c]identical sequences.

Sequences were submitted to the European Molecular Biology (EMBL) database and are available under accession numbers AM945858 – AM945867 (*FUM1*) and AM945868 – AM945876 (*FUM8*).

After removal of primer sequences, nucleotide identity within the eleven 484-bp *F. proliferatum FUM1* fragments (including the database reference sequence acc. AY577458 and sequences of two individual clones each from isolates Fpro-227 and Fpro-241), ranged between 99.1 and 100%. Comparison among Fpro-227 and Fpro-241 *FUM1* sequences revealed identities of 99.3 and 99.7% at the clone level respectively. A parallel analysis of *FUM8* fragments derived from nine different *F. proliferatum* isolates (10 sequences, the reference acc. AY577451 and two individual clones received from Fpro-241) produced results analogous to results obtained from the *FUM1* sequences. Comparisons among the 758-bp *FUM8* fragments from different isolates exhibited nucleotide identities between 99.0 and 100%, while sequences from individual clones of isolate Fpro-241 were 99.8% identical. As outlined in Table 2, minor sequence variability of 0.3% (*FUM1*, 488 bp) and sequence identity of the *FUM8* gene (775 bp) respectively, was found within the corresponding fragments of reference sequences from different *F. verticillioides* strains stored in the database. Taken together, sequence variations of the *F. proliferatum* population occurring in asparagus are also present in *FUM1* and *FUM8* genes, reflecting the genome diversity within this species obtained by RAPD- and DAF-PCR fingerprints. However, fingerprint grouping and sequence variability of *FUM* genes within the *F. proliferatum* strains were not linked, because isolates belonging to the same fingerprint cluster in some cases showed higher sequence variability of *FUM* genes than *F. proliferatum* strains obtained from different clusters (data not shown).

Comparison of *FUM1* and *FUM8* gene fragments with sequences from eight different *Fusarium* spp. showed that interspecific sequence variability was higher for these fragments than was intraspecific variability. Fumonisin gene fragments from *F. proliferatum* showed closest relation to the *FUM1* and *FUM8* of *F. globosum* and *Fusarium fujikuroi*, which shared 96.0% or more of *FUM1* or *FUM8* sequence. In contrast, *Fusarium nygamai* and *F. verticillioides* were the most distantly related species, exhibiting identities of only 84% (*FUM1*) and 77% (*FUM8*) as indicated in Table 2. Notably, *FUM8* gene fragments exhibited overall higher sequence variability than *FUM1* genes among *Fusarium* spp., presumably as a result of the longer fragment length of *FUM8*. Nevertheless, phylogenetic reconstructions by neighbour-joining, maximum-likelihood and maximum-parsimony algorithms showed that clustering of *Fusarium* spp. according to *FUM1* and *FUM8* nucleotide diversity was consistent with species complexes as defined by O'Donnell et al. (1998). Differentiation of species was supported by high bootstrap values, generally above 950, as shown in Fig. 4 for the maximum-parsimony trees of nucleotide sequences of *FUM* genes. Based on these analyses *F. proliferatum* isolates clustered together with other members of the *Gibberella fujikuroi* species complex clade 3 (*F. globosum*, *F. fujikuroi)* and were clearly distinguished from *Fusarium* spp. representing other *G. fujikuroi* species complexes (*Fusarium antophilum*, *F. nygamai*, *F. verticillioides*) and the single *F. oxysporum* isolate, which is the sole member of the *F. oxysporum* species complex available.

Discussion

Fusarium proliferatum isolates, which were recovered from asparagus spears from different locations in Austria and Germany, exhibited considerable genetic variability. RAPD and DAF-PCR analyses indicated that fourteen different fungal haplotypes could be distinguished. Molecular phylogenetic analyses of strains from diverse hosts and geographic areas initiated by Geiser et al. (Desjardins, 2003) confirmed high diversity within *F. proliferatum* (G. *intermedia*). However,

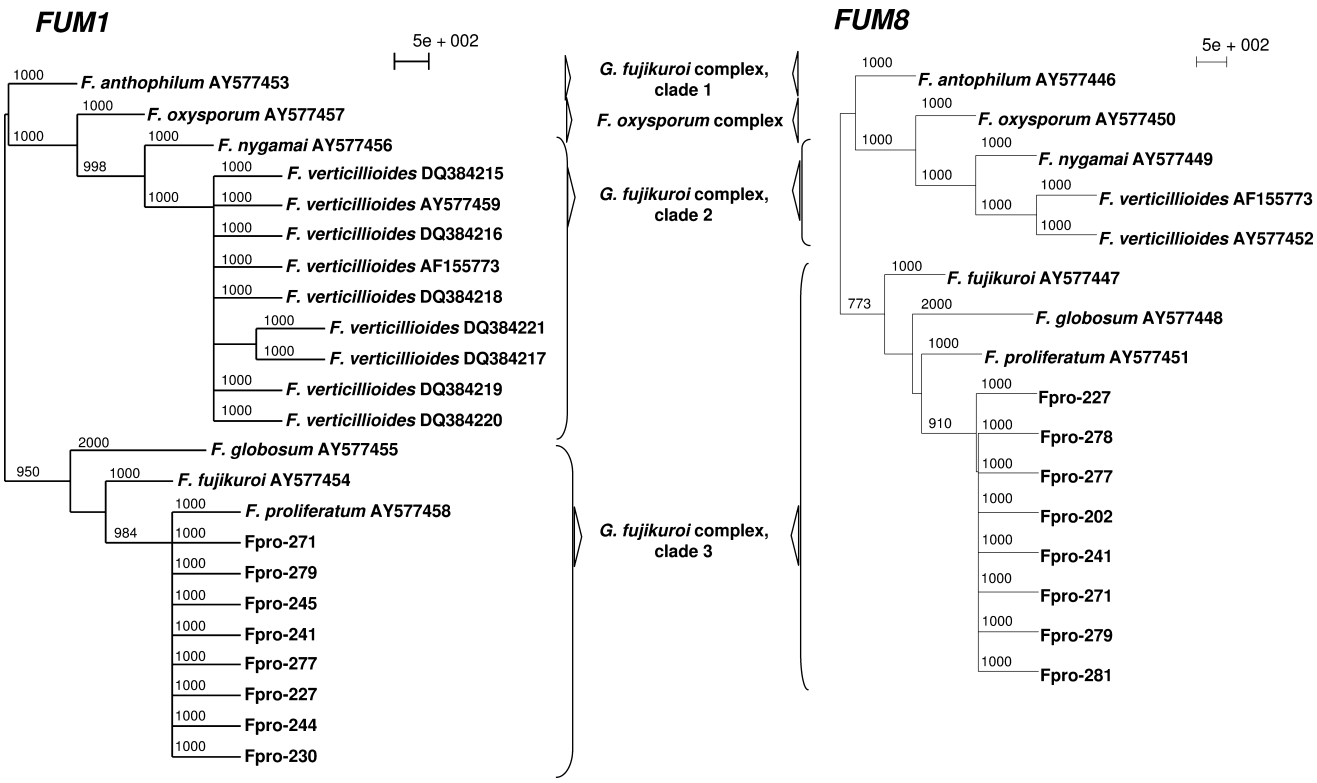

Fig. 4 Maximum-parsimony phylograms of *FUM1* and *FUM8* nucleotide fragments obtained from *Fusarium proliferatum* strains in comparison with corresponding *Fusarium* spp. sequences from the database (indicated by accession numbers behind the *Fusarium* species). Species complexes according to O'Donnell et al. (1998) are indicated in the middle. Bootstrap values ($n = 1000$ repetitions) over 700 are indicated on branch nodes

no correlation between fingerprint group, geographic origin or presence of *FUM* genes or fumonisin B production was found. Missing relationships between fingerprint groups and origin of isolates or other characteristic features are often described for different *Fusarium* species, which causes various plant diseases. For instance, Abd-Elsalam et al. (2003) were able to correlate diverse fingerprint patterns of *Fusarium semitectum* isolates with morphological characteristics of fungal cultures, but found no correlation with host genotype or geographic origin. Even though some *Fusarium avenaceum* isolates originating from same fields could be grouped according to similarity of RAPD-PCR patterns, a study by Satyaprasad et al. (2000) could not demonstrate the existence of a relationship between pathogenicity, vegetative compatibility group or genome diversity determined by RAPD-PCR. However, this might have been due to the isolate characteristics chosen for analysis, which were descriptive phenomena or measured phenotypic traits. The latter are often regulated by multiple genes, thus making a correlation with RAPD-PCR markers difficult.

Despite the well-known diversity of the fungal genome within *F. proliferatum*, *FUM1*- and *FUM8*-genes could be detected in all isolates included in this study. Confirmation of the presence of these genes is indicative of the ability of these strains to produce fumonisins. Consequently, these isolates may have been responsible for the contamination of asparagus spears with B type fumonisins reported by Weber et al. (2006) and Gossmann et al. (2007). These findings support results obtained by Jeney et al. (2004), Proctor et al. (2002) and Rheeder et al. (2002), who contended that nearly all wildtype *F. proliferatum* isolates are able to produce fumonisins. For instance, *in vitro* analyses of 38 *F. proliferatum* (*G. intermedia*) strains obtained from prairie grasses in Kansas, revealed that all, but one isolate produced high levels of total fumonisins, often above 1000 μg/g (Leslie et al., 2004).

In our study, uniform restriction patterns for all *FUM1* and all except one *FUM8* sequence fragments were generated after digestion with restriction enzymes, enabling differentiation of *F. proliferatum* strains from other fumonisin producing species included in this study, in accordance with RFLP analysis *in silico*. Normally, genes and intergenic spacer regions of rDNA sequences (Mirete et al., 2004) are targeted for PCR-RFLP differentiation of *Fusarium* species (Lee et al., 2000; Llorens et al., 2006) because they are taxonomically relevant. For instance, Baayen et al. (2000) developed a PCR-RFLP analysis that utilized Internal transcribed spacer (ITS) regions to discriminate *F. proliferatum*, *Fusarium redolens* and *F. oxysporum* isolates obtained from asparagus. Similarly, the present PCR-RFLP analysis of amplified *FUM1* and *FUM8* genes may be a suitable method for identifying *F. proliferatum* isolates to the species level.

Confirmation of the specificity of the method presented here requires further testing, particularly of isolates of other *Fusarium* spp., particularly the fumonisin-producing, asparagus-colonizing fungi *F. oxysporum*, *F. redolens* and *Fusarium subglutinans*, as well as other Fusaria isolated from alternative host plants.

Intraspecific sequence diversity of *F. proliferatum* isolates from asparagus had previously only been determined for a few strains obtained from various plantings in North America. Analyzing EF1-α sequences from isolates obtained during the last week of asparagus harvest, Vujanovic et al., 2006 discovered sequence similarity around of 96% within the species, which is similar to the intraspecific variability of partial *FUM1* and *FUM8* sequences determined in our investigation. In a comparison of the first 1020 bp of the *FUM1* of six *F. verticillioides* strains isolated from corn and sorghum, da Silva et al. (2007) found that sequence diversity of certain strains varies between 0.2 and 0.3%. These data correspond to values we obtained in our intraspecific comparison of *F. proliferatum* strains, probably because the smaller (488 bp) *FUM1* fragment we analyzed is enclosed in the sequence used by da Silva et al. (2007). However, da Silva et al.'s speculation approximately a correlation between *FUM1* sequence diversity and levels of fumonisin production seems not to apply to *F. proliferatum* isolates from asparagus. Despite the presence of *FUM1*, the detected fumonisin levels in asparagus spears infected with the isolates included in this study, as determined by Gossmann et al. (2007) and Weber et al. (2006), were similarly low (Table 1).

The analyzed 488 bp of *FUM1* are covering only 6% of the whole polyketide synthase gene, which is presumably around 8.1 kb in length, estimated by the *F. verticillioides* sequence in the database; the analyzed 758 bp covered 26% of the *FUM8* gene (2.9 kb). Mean sequence similarities of 84.3% (*FUM1*) and 77.2% (*FUM8*) respectively, which were estimated for sequenced *F. proliferatum* and *F. verticillioides* gene fragments, are obviously representative of variability of the complete genes, because Waalwijk et al. (2004) reported 85 and 77% sequence identity, respectively of the whole *FUM1* and *FUM8* open reading frames.

Degree of sequence conservation of *FUM1* and *FUM8* will enable the development of primers for general detection of fumonisin producing fusaria in asparagus, but the interspecific diversity of these genes is also suitable for species specific differentiation of this *Fusarium* spp., which may be achieved for one by generation of species specific primers or as well by application of PCR-RFLP method. *FUM1* primers have been developed for instance by Bluhm et al. (2002). Yet, primer sequences were targeting fumonisin producing species infecting cereals and based upon *F. verticillioides* sequences. More universal primers have been developed by Gonzales-Jaen et al. (2004), allowing detection of *FUM1* in the species *F. verticillioides* and *F. proliferatum*. Suitability of the *FUM1* gene for detection of fumonisin producing fusaria isolated from freshly harvested maize kernels has most recently been shown by Sreenivasa et al. (2007). Described primers were able to detect *FUM1* from *F. verticillioides*, *F. proliferatum* and *F. antophilum*. However, amplified products with a length of 183 bp would require sequencing for identification of the originating *Fusarium* species, because it may be to short for direct species discrimination by development of further species specific PCR primers. Proctor et al. (2004) published *F. verticillioides* derived *FUM1* and *FUM8* primers suitable for detection of these genes in certain isolates belonging to the species *F. anthophilum F. fujikuroi, F. globosum, F. nygamai* and *F. oxysporum*. Amplified sequences covered approx. 680 nucleotides of the *FUM1* and 1030 bp of the *FUM8* coding region. As primers presented in this study are enclosed within the fragments generated by the oligonucleotides describend by Proctor et al. (2004), they may be suitable in combination for a sensitive nested PCR approach for the detection of fusaria in food.

Fusarium proliferatum, *F. verticillioides* and most other fumonisin producing species, are members of the *G. fujikuroi* species complex, a group of at least 29 *Fusarium* species that corresponds to *Fusarium* section *Liseola* (Nirenberg and O'Donnell, 1998). These species groups were clearly distinguishable by phylogenetic analysis of *FUM1* and *FUM8* sequences. *Fusarium proliferatum* is phylogenetically closely related to *F. fujikuroi* and *F. globosum* within clade 3 of the *G. fujikuroi* species complex as was shown by multilocus sequence comparisons of β-tubulin, ITS and rDNA genes (O'Donnell et al., 1998). This close relationship was also confirmed by phylogenetic analysis of *FUM1* and *FUM8* sequences in this study, where these three species clustered as a separate sister group. However, grouping of *F. oxysporum* isolate within the *F. verticillioides*, *F. antophilum* lineage was contradictory to results obtained by O'Donnell et al. (1998), because this species does not belong to the *G. fujikuroi* complex, although it is closely related. However, it confirmed findings of Waalwijk et al. (2004), who proposed that the fumonisin biosynthetic cluster is paraphyletic, which particularly attributes to the *F. oxysporum* complex and includes strains pathogenic in asparagus and other causal agents of root and bulb diseases (Baayen et al., 2000).

Concluding, genome wide variability of *F. proliferatum* is mirrored by *FUM1* and *FUM8* gene diversity, but obviously not in the presence or absence of the fumonisin biosynthetic gene cluster within this species. The detection of marker genes for the fumonisin metabolic pathway is considered a valid method to screen for mycotoxic fungi (Russell and Paterson, 2006) and may serve the purpose to determine their potential of toxin production. Additionally, genetic diversity of *FUM* genes may enable identification of *Fusarium* species. To serve these goals, genes, primers and targeted sequences have to be chosen with care. As the *FUM8* sequence exhibited higher variability as the polyketide

synthase coding gene, this gene may prove as a more suited target sequence for species specific detection of *Fusarium* spp. in asparagus. However, as for other fumonisin specific primer sets developed to date, suitability of primers used in this study have to be tested further, to detect fumonisin producing fusaria directly in asparagus plant material. Moreover, corresponding sequences from other fumonisin producing fungi occurring in asparagus have to be determined and evaluated.

Acknowledgements

We thank Ralph-Martin Lange (Alberta Research Council, Vegreville, Canada) for valuable comments and for proofreading the manuscript.

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