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Fusarium temperatum sp. nov. from maize, an emergent species closely related to *Fusarium subglutinans*

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Abstract: A large number of *Fusarium* isolates closely related to *F. subglutinans* were collected from maize in Belgium. We used a robust polyphasic approach to describe a new biological species, *Fusarium temperatum*, within the *Gibberella fujikuroi* species complex. *F. temperatum* can be distinguished from *F. subglutinans* and from other *Fusarium* species within the *Gibberella fujikuroi* species complex with AFLP fingerprint profile, differences in the translation elongation factor 1-α and β-tubulin DNA sequence and interspecies mating compatibility analyses. Intraspecies mating compatibility suggests that sexual reproduction might be common for field isolates of *F. temperatum*, and reliable female fertile mating population tester strains were proposed for this heterothallic species.

Key words: AFLP, biological species, β-tubulin, *Fusarium subglutinans*, *Gibberella fujikuroi* species complex, maize, mating population, phylogeny, taxonomy, translation elongation factor 1-α

INTRODUCTION

Fusarium subglutinans (Wollenw. & Reinking) P.E. Nelson, Toussoun & Marasas (teleomorph: *Gibberella subglutinans* [E.T. Edwards] P.E. Nelson, Toussoun & Marasas) is an important pathogen of maize, common in temperate regions (Leslie and Summerell 2006). The species belonging to the *Gibberella fujikuroi* species complex (GFSC) is associated with stalk and ear rot and also can be recovered from symptomless plants or seeds (Edwards 1935, Kabeere et al. 1997, White 1999). Furthermore *F. subglutinans* is a toxigenic species that can produce moniliformin, fusaproliferin, fusaric acid and beauvericin (Lew et al. 1996, Bottalico 1998, Desjardins 2006).

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Species description in the GFSC is based on a polyphasic approach combining morphological species recognition (MSR), biological species recognition (BSR) with diagnostic sexual crosses and phylogenetic species recognition (PSR) using DNA sequence polymorphisms (Taylor et al. 2000, Kvas et al. 2009). Based on these concepts, *F. subglutinans* isolated mainly from maize and previously described as mating population E (MP-E) was separated from morphologically similar species, such as *F. circinatum* Nirenberg & O'Donnell (MP-H) isolated from pine, *F. sacchari* (E.J. Butler & Hafiz Khan) W. Gams (MP-B) isolated from sugarcane, *F. guttiforme* Nirenberg & O'Donnell isolated from pineapple and *F. mangiferae* Britz, M.J. Wingf. & Marasas that causes mango malformation (Leslie 1991, 1995; Nirenberg and O'Donnell 1998; O'Donnell et al. 1998a; Britz et al. 1999, 2002). *Fusarium* species of the GFSC were separated into three clades (the so-called American, African and Asian clades) with *F. subglutinans* included in the American clade, according to the phylogeographic study by O'Donnell et al. (1998a). Furthermore application of the PSR revealed that species *F. subglutinans* is subdivided into two main phylogenetically distinct groups (1 and 2) that appear to be reproductively isolated in nature, even though interfertile crosses occurred under laboratory conditions. These *F. subglutinans* groups might be in the process of divergence (Desjardins et al. 2000; Steenkamp et al. 2001, 2002). Within group 1 the strain *Fusarium* sp. NRRL 25622 (= MRC 1077, = MUCL 51714) isolated from maize in South Africa and originally identified as *F. subglutinans* (Viljoen et al. 1997) has been taxonomically problematic. It was resolved as phylogenetically distinct from *F. subglutinans* (O'Donnell et al. 2000) but also reported to be sexually compatible with one of the *F. subglutinans* mating type tester strains (Steenkamp et al. 1999).

Among the 5660 *Fusarium* strains belonging to 23 species isolated from maize during a 3 y survey (2005–2007) in Belgium, 285 *Fusarium* strains morphologically very similar to *F. subglutinans* were collected. Strikingly only nine strains were identified as *F. subglutinans* on the basis of additional molecular data while the 276 remaining strains presented 99–100% translation elongation factor 1-α (*EF-1α*) sequence similarity to the *Fusarium* sp. NRRL 25622 strain. The objective of the research was to define the taxonomical rank of those strains within the GFSC and

particularly their relationship with *F. subglutinans* and *Fusarium* sp. NRRL 25622. We used a polyphasic approach based on (i) amplified fragment length polymorphisms (AFLPs) to characterize intraspecific variability, (ii) PSR using parsimony and Bayesian analyses, (iii) BSR using sexual crosses to assess their fertility and (iv) MSR. This approach resulted in the formal description of a new species, *Fusarium temperatum*.

MATERIALS AND METHODS

Fungal isolates and culture conditions.—We analyzed 30 *F. temperatum* strains and three *F. subglutinans* strains isolated from maize in Belgium (TABLE I). Tester strains of *F. verticillioides* (Sacc.) Nirenberg (MP-A), *F. sacchari* (MP-B), *F. subglutinans* (MP-E), *F. circinatum* (MP-H) and *F. konzum* Zeller, Summerell & J.F. Leslie (MP-I) were obtained from the Fungal Genetics Stock Center (University of Missouri, USA) and strain NRRL 25622 was kindly provided by K. O'Donnell, from the Agricultural Research Service Culture Collection (U.S. Department of Agriculture). Monoconidial strains are cryopreserved and maintained in tubes on SNA (Leslie and Summerell 2006) under mineral sterile oil at the BCCM™/MUCL collection. For colony morphology and growth, as well as for conidiogenesis analyses, strains were grown respectively on potato dextrose agar (PDA; Sharlau, Barcelona, Spain) and SNA. Observations were made as described by van Hove et al. (In press).

DNA extraction.—Fungal isolates were grown in the dark at 25 C 5 d in a 50 mL malt extract 2% broth medium (20 g of malt extract L^{-1}, Duchefa, Haarlem, the Netherlands) on a rotary shaker (100 rpm). Mycelium was harvested by centrifugation, and the pellets were lyophilized and stored at −20 C. The lyophilized mycelia were disrupted in a MagNA Lyser cell disrupter (Roche Diagnostics GmbH, Mannheim, Germany). Fungal DNA was extracted and purified with the Invisorb Spin Plant MiniKit (Invitek GmbH, Berlin, Germany) according to the manufacturer's recommendations. Purified DNA was quantified with a Bio Photometer (Eppendorf, Hamburg, Germany) and stored at −30 C.

Amplified fragment length polymorphisms (AFLP).—AFLPs (Vos et al. 1995) were generated as described by Voyron et al. (2009). Pre-amplification was performed with the core *MseI* site primer (M) and *EcoRI* site primer (E). Selective PCR amplification was performed with D4-labeled *EcoRI* primers with a two-base selection (E-AC and E-GG) and unlabeled *MseI* primers with a two-base selection (M-CC and M-CG). Four combinations, E-AC/M-CC, E-AC/M-CG, E-GG/M-CC and E-GG/M-CG, were tested.

Amplified fragments were analyzed by capillary electrophoresis on the CEQ™ 2000 Genetic Analysis System with the Fragment Analyses Module software (Beckman Coulter, Fullerton, California). AFLP data were viewed as fingerprinting profiles with Genographer 1.6.0 (Benham, Montana State University, Bozeman, Montana). All AFLP bands 100–660 bp were scored manually as present or absent and checked for repeatability. We assumed that bands of the same molecular size in different individuals were identical (homologous characters). The binary matrix was imported into FreeTree 9.1.50 software (Pavlieek et al. 1999) to perform a cluster analysis. Dice coefficient (Nei and Li 1979) was used to calculate pairwise UPGMA genetic distances among strains. The topologies of the trees were assessed by bootstrapping with 1000 replications.

DNA amplification, sequencing and phylogenetic analyses.—Amplification of the *EF-1α* gene strains was carried out with PCR primers EF1 and EF2 using the amplification conditions of O'Donnell et al. (1998b). Portions of the β-tubulin gene were amplified with PCR primers T1 and T22 under PCR conditions described in O'Donnell and Cigelnik (1997). All PCR were carried out in a TGradient thermocycler (Biometra GmbH, Goettingen, Germany). PCR products were purified with the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and sequenced in both directions in a 3100 Genetic Analyzer (Applied Biosystems, USA). Sequences were edited with Sequencher 4.8 (Gene Codes Corp., Ann Arbor, Michigan). DNA sequences generated in our study were deposited in GenBank under accessions numbers HM067684–HM067699. β-tubulin and *EF-1α* gene sequences from 36 *Fusarium* species in the GFSC were obtained from GenBank (TABLE II). All sequences were aligned with Clustal W2 (Larkin et al. 2007) and manually adjusted with Squint Alignment Editor 1.02 (Goode, University of Auckland, New Zealand). Sequence data from *EF-1α* and β-tubulin genes were analyzed separately as well as combined because they were shown to represent homogenous partitions (O'Donnell et al. 1998a). Final sequence alignments are available at TreeBASE, accession number S10749, http://purl.org/phylo/treebase/phylows/study/TB2:S10749.

Maximum parsimony trees were inferred with PAUP* 4.0b10 (Swofford 2000) with the heuristic search option with 1000 random addition sequences, tree-bisection-reconnection branch swapping and MULTREES effective. Alignment gaps were treated as a fifth character (newstate) and 1000 parsimony bootstrap replications were conducted to test clade support. Consistency index (CI) and retention index (RI) were calculated to obtain the amount of homoplasy in the dataset.

Bayesian phylogenetic analyses were inferred with a Metropolis-coupled Markov chain Monte Carlo methodology as implemented in MrBayes 3.1 (Ronquist and Huelsenbeck 2003) to calculate posterior probabilities. Models that best fit the provided sequence dataset were evaluated with Modeltest 3.06 (Posada and Crandall 1998). The general time reversible model (GTR + I + G) and the Hasegawa-Kishino-Yano (HKY + G) were selected respectively for Bayesian analyses of the *EF-1α* and β-tubulin nucleotide partitions. Two concurrent analyses of four chains (one cold and three heated) were run 2×10^6 generations, ensuring analyses were not trapped at local optima, with random starting trees, and sampled every 1000 generations. Trees collected before the stable likelihood

TABLE I. *Fusarium* strains used in this study

Clade[a]/species	Isolate number[b]	*MAT1* allele	Origin	Host/Substrate	References
American					
F. temperatum	MUCL 51714 (NRRL 25622, MRC 1077)	2	South Africa	*Zea mays*	Steenkamp et al. 1999
	MUCL 52436	1	Belgium	*Zea mays*	this study
	MUCL 52437	1	Belgium	*Zea mays*	this study
	MUCL 52438	2	Belgium	*Zea mays*	this study
	MUCL 52439	1	Belgium	*Zea mays*	this study
	MUCL 52440	1	Belgium	*Zea mays*	this study
	MUCL 52441	1	Belgium	*Zea mays*	this study
	MUCL 52442	1	Belgium	*Zea mays*	this study
	MUCL 52443	2	Belgium	*Zea mays*	this study
	MUCL 52444	2	Belgium	*Zea mays*	this study
	MUCL 52445	1	Belgium	*Zea mays*	this study
	MUCL 52446	2	Belgium	*Zea mays*	this study
	MUCL 52447	2	Belgium	*Zea mays*	this study
	MUCL 52448	2	Belgium	*Zea mays*	this study
	MUCL 52449	1	Belgium	*Zea mays*	this study
	MUCL 52450	2	Belgium	*Zea mays*	this study
	MUCL 52451	2	Belgium	*Zea mays*	this study
	MUCL 52452	1	Belgium	*Zea mays*	this study
	MUCL 52453	2	Belgium	*Zea mays*	this study
	MUCL 52454	1	Belgium	*Zea mays*	this study
	MUCL 52455	1	Belgium	*Zea mays*	this study
	MUCL 52456	1	Belgium	*Zea mays*	this study
	MUCL 52457	1	Belgium	*Zea mays*	this study
	MUCL 52458	1	Belgium	*Zea mays*	this study
	MUCL 52459	2	Belgium	*Zea mays*	this study
	MUCL 52460	2	Belgium	*Zea mays*	this study
	MUCL 52461	1	Belgium	*Zea mays*	this study
	MUCL 52462	2	Belgium	*Zea mays*	this study
	MUCL 52463	1	Belgium	*Zea mays*	this study
	MUCL 52464	1	Belgium	*Zea mays*	this study
	MUCL 52465	2	Belgium	*Zea mays*	this study
F. subglutinans	MUCL 43484 (FGSC 7616)	1	United States	*Zea mays*	Nelson et al. 1983
	MUCL 43485 (FGSC 7617)	2	United States	*Zea mays*	Nelson et al. 1983
	MUCL 52466	1	Belgium	*Zea mays*	this study
	MUCL 52467	1	Belgium	*Zea mays*	this study
	MUCL 52468	2	Belgium	*Zea mays*	this study
F. circinatum	MUCL 47028 (FGCS 9022)	1	South Africa	*Pinus* sp.	Britz et al. 1999
	MUCL 47029 (FGSC 9023)	2	South Africa	*Pinus* sp.	Britz et al. 1999
F. konzum	MUCL 47030 (FGSC 8910)	1	United States	*Amiropogon* sp.	Zeller et al. 2003
	MUCL 47031 (FGSC 8911)	2	United States	*Amiropogon* sp.	Zeller et al. 2003
African					
F. verticillioides	MUCL 43478 (FGSC 7600)	1	United States	*Zea mays*	Gerlach and Nirenberg 1982
	MUCL 43479 (FGSC 7603)	2	United States	*Zea mays*	Gerlach and Nirenberg 1982
Asian					
F. sacchari	MUCL 43481 (FGSC 7611)	1	United States	Laboratory cross	Leslie et al. 2005
	MUCL 43480 (FGSC 7610)	2	United States	Laboratory cross	Leslie et al. 2005

[a] Clades of the GFSC as reported in O'Donnell et al. 1998a.

[b] FGSC = Fungal Genetics Stock Center, Kansas City, USA; MRC = Medical Research Council, Tygerberg, South Africa; MUCL = Mycothèque de l'Université catholique de Louvain, Louvain-la-Neuve, Belgium; NRRL = Agricultural Research Service Culture Collection, Peoria, USA.

TABLE II. GenBank accession numbers of *Fusarium* spp. of the GFSC used to generate the PSRphylogram

Species	Culture collection[a]	Origin	Host/Substrate	β-tubulin	EF 1-α
F. acutatum	NRRL 13308	India	unknown	U34431	AF160276
F. anthophilum	NRRL 13602	Germany	*Hippeastrum sp.*	U61541	AF160292
F. bactridioides	NRRL 20476	United States	*Cronartium conigenum*	U34434	AF160290
F. begoniae	NRRL 25300	Germany	*Begonia elatior*	U61543	AF160293
F. brevicatenulatum	NRRL 25446	Madagascar	*Striga asiatica*	U61545	AF160265
F. bulbicola	NRRL 13618	Netherlands	*Nerine bowdenii*	U61546	AF160294
F. circinatum	NRRL 25331	United States	*Pinus radiata*	U61547	AF160295
F. concentricum	NRRL 25181	Costa Rica	*Musa sapientum*	U61548	AF160282
F. denticulatum	NRRL 25302	United States	*Ipomoea batatas*	U61550	AF160269
F. dlaminii	NRRL 13164	South Africa	*Zea mays*	U34430	AF160277
F. fractiflexum	NRRL 28852	Japan	*Cymbidium* sp.	AF160315	AF160288
F. fujikuroi	NRRL 13566	Taiwan	*Oryza sativa*	U34415	AF160279
F. globosum	NRRL 26131	South Africa	*Zea mays*	U61557	AF160285
F. guttiforme	NRRL 22945	England	*Ananas comosus*	U34420	AF160297
F. inflexum	NRRL 20433	Germany	*Vicia faba*	U34435	AF8479
F. konzum	MRC 8544	United States	*Sorghastrum nuttans*	EU220234	EU220235
F. lactis	NRRL 25200	United States	*Ficus carica*	U61551	AF160272
F. mangiferae	NRRL 25226	India	*Mangifera indica*	U61561	AF160281
F. napiforme	NRRL 13604	South Africa	*Pennisetum typhoides*	U34428	AF160266
F. nygamai	NRRL 13448	Australia	*Sorghum bicolor*	U34426	AF160273
F. oxysporum	NRRL 22902	United States	*Pseudotsuga menziesii*	U34424	AF160312
F. phyllophilum	NRRL 13617	Italy	*Dracaena deremensis*	U34432	AF160274
F. proliferatum	NRRL 22944	Germany	*Cattleya* sp.	U34416	AF160280
F. pseudoanthophilum	NRRL 25206	Zimbabwe	*Zea mays*	U61553	AF160264
F. pseudocircinatum	NRRL 22946	Ghana	*Solanum* sp.	U34427	AF160271
F. pseudonygamai	NRRL 13592	Nigeria	*Pennisetum typhoides*	U34421	AF160263
F. ramigenum	NRRL 25208	United States	*Ficus carica*	U61554	AF160267
F. sacchari	NRRL 13999	India	*Saccharum officinarum*	U34414	AF160278
Fusarium sp.	NRRL 25622	South Africa	*Zea mays*	AF160317	AF160301
F. temperatum	MUCL 52436	Belgium	*Zea mays*	HM067692	HM067684
F. temperatum	MUCL 52443	Belgium	*Zea mays*	HM067693	HM067685
F. temperatum	MUCL 52445	Belgium	*Zea mays*	HM067694	HM067686
F. temperatum	MUCL 52450	Belgium	*Zea mays*	HM067695	HM067687
F. temperatum	MUCL 52451	Belgium	*Zea mays*	HM067696	HM067688
F. temperatum	MUCL 52454	Belgium	*Zea mays*	HM067697	HM067689
F. temperatum	MUCL 52462	Belgium	*Zea mays*	HM067698	HM067690
F. sterilihyphosum	CML 283	Brazil	*Mangifera indica*	DQ445780	DQ452858
F. subglutinans	NRRL 22016	United States	*Zea mays*	U34417	AF160289
F. subglutinans	MUCL 52468	Belgium	*Zea mays*	HM067699	HM067691
F. succisae	NRRL 13613	Germany	*Succisa pratensis*	U34419	AF160291
F. thapsinum	NRRL 22045	South Africa	*Sorghum bicolor*	U34418	AF160270
F. udum	NRRL 22949	Germany	unknown	U34433	AF160275
F. verticillioides	NRRL 22172	Germany	*Zea mays*	U34413	AF160262
F. xylarioides	NRRL 25486	Ivory Coast	*Coffea sp.*	AY707118	AY707136

[a] CML = Coleção Micológica de Lavras, Universidade Federal de Lavras, Lavras, Brazil; MRC = Medical Research Council, Tygerberg, South Africa; MUCL = Mycothèque de l'Université catholique de Louvain, Louvain-la-Neuve, Belgium; NRRL = Agricultural Research Service Culture Collection, Peoria, USA.

value point were discarded as burn-in. A majority rule consensus tree was constructed from the remaining trees, and the Bayesian posterior probabilities of clades were calculated. *Fusarium oxysporum* and *F. inflexum* were used as outgroups (O'Donnell et al. 1998a).

Mating type specific PCR and crossing procedure.—Mating type idiomorphs (*MAT1-1* or *MAT1-2*) were identified with PCR-based assays as described by Steenkamp et al. (2000) and Lepoint et al. (2005). Crosses were conducted as described in Klittich and Leslie (1988), except that after fertilization cultures were maintained under an 8 h day/16 h night cycle. On the basis of the genetic analyses (AFLP) 10 *F. temperatum* strains were crossed as male with the tester strains available for the three well characterized biological species belonging to the American clade, *F. subglutinans*, *F.*

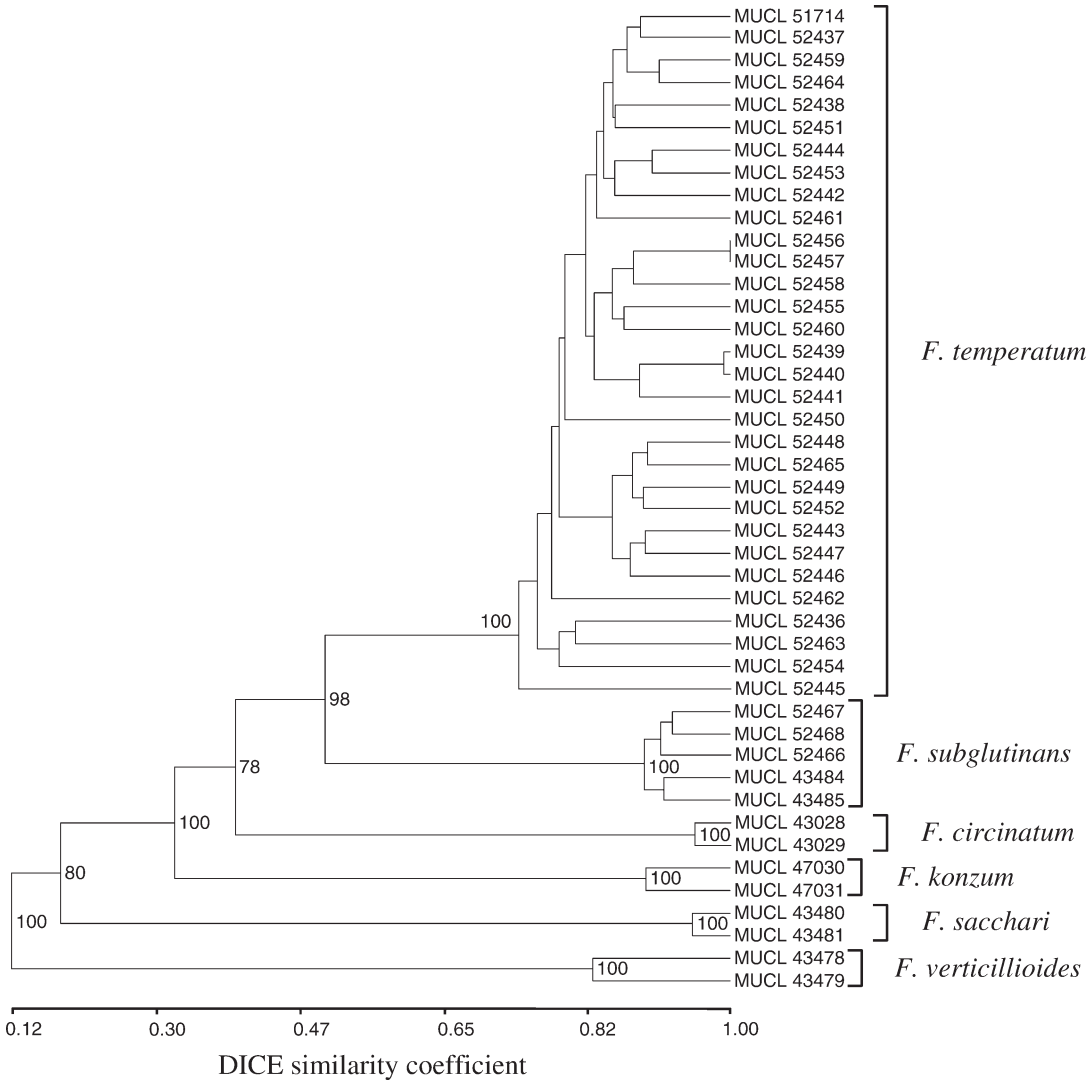

FIG. 1. AFLP dendrogram generated from UPGMA cluster analysis showing the genetic similarities (Dice similarity coefficient) among the *Fusarium* species in this study. Support from 1000 bootstrap iterations is indicated for the clusters with values above 70%. MUCL = Mycothèque de l'Université catholique de Louvain, Louvain-la-Neuve, Belgium.

circinatum and *F. konzum*. In addition those strains and *Fusarium* sp. NRRL 25622 strain were self-crossed as well as intercrossed in all possible *MAT1-1* × *MAT1-2* combinations, including both male and female fertility. *F. verticillioides* (MP-A) testers strains were crossed and used as internal positive controls. Crosses were conducted in triplicate in at least two experiments. Fertility was confirmed by observation of a cirrhus atop the perithecium and by microscopic observation of mature asci and ascospores.

RESULTS

AFLP fingerprinting and divergence among species.—A total of 311 AFLP bands 100–660 bp were scored after amplification with the four primer pair combinations. The number of polymorphic fragments per primer combination were 72–80.

Six distinct clusters were identified among the diverse GFSC isolates analyzed, according to the UPGMA analysis (FIG. 1). The first cluster included the strain NRRL 25622 (= MUCL 51714) and 30 newly characterized isolates described herein as *F. temperatum*. The second cluster included the two tester strains of *F. subglutinans* and the three *F. subglutinans* strains from Belgium. Both tester strains of *F. circinatum*, *F. konzum*, *F. sacchari* and *F. verticillioides* are included in the four other clusters. The distinctness of all six clusters was supported by bootstrap values of 78–100% in 1000 replicates.

The genetic similarity estimated with the Dice coefficient between *F. temperatum* and *F. subglutinans* was about 51% and that between *F. temperatum* and the remaining *Fusarium* species tested was less

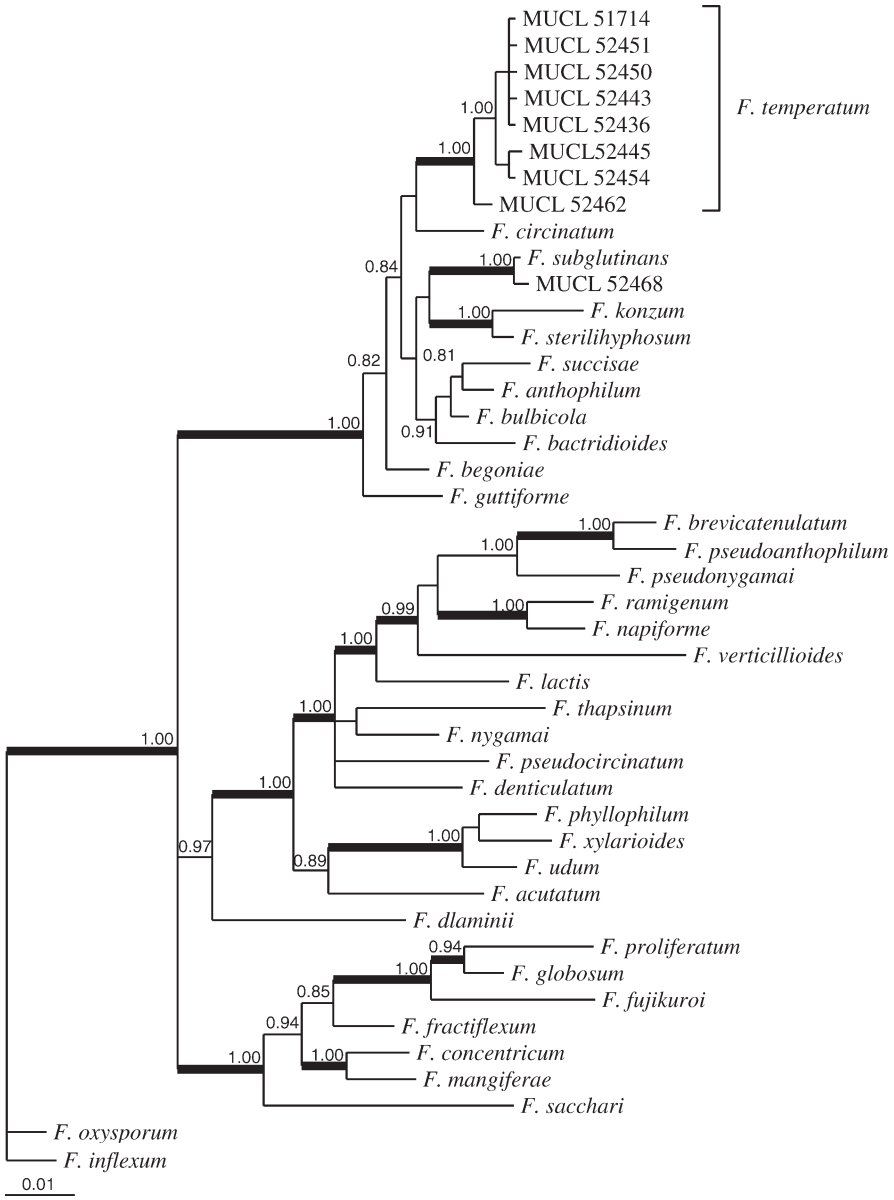

FIG. 2. Bayesian inference tree based on partial sequences of β-tubulin (−lnL = 1753.49) and EF-1α (−lnL = 3244.22) loci. Values at branch nodes indicate branch support with posterior probabilities (PP; values ≥ 0.80 shown) and branches in boldface = bootstrapping percentages based on maximum parsimony analysis ≥ 70%. Bar represents the substitutions expected per site.

than 40%. In the *F. temperatum* cluster genetic similarity among the 31 strains was 74–100%. A total of 30 unique AFLP fingerprint haplotypes were observed among the 31 *F. temperatum* strains. The two strains (MUCL 52456 and MUCL 52457) presenting the same haplotype were collected in the same field.

Phylogeny.—On the basis of the AFLP results strain NRRL 25622 and seven strains (MUCL 52436, MUCL 52443, MUCL 52445, MUCL 52450, MUCL 52451, MUCL 52454 and MUCL 52462) representative of the genetic variation within the *F. temperatum* cluster were selected for phylogenetic analyses. Amplified DNA sequences of the β-tubulin and *EF-1α* genes were aligned among the sequences available from GenBank. The three *F. subglutinans* strains shared identical combined sequences and were represented by MUCL 52468 (TABLE II). The aligned *EF-1α* gene sequences of the *F. temperatum* strains were 619 bp long. Six nucleotides were polymorphic (1%) and no site had more than two different nucleotide character states. Strains MUCL 52450 and MUCL 52451 had the same sequence as strain NRRL 25622

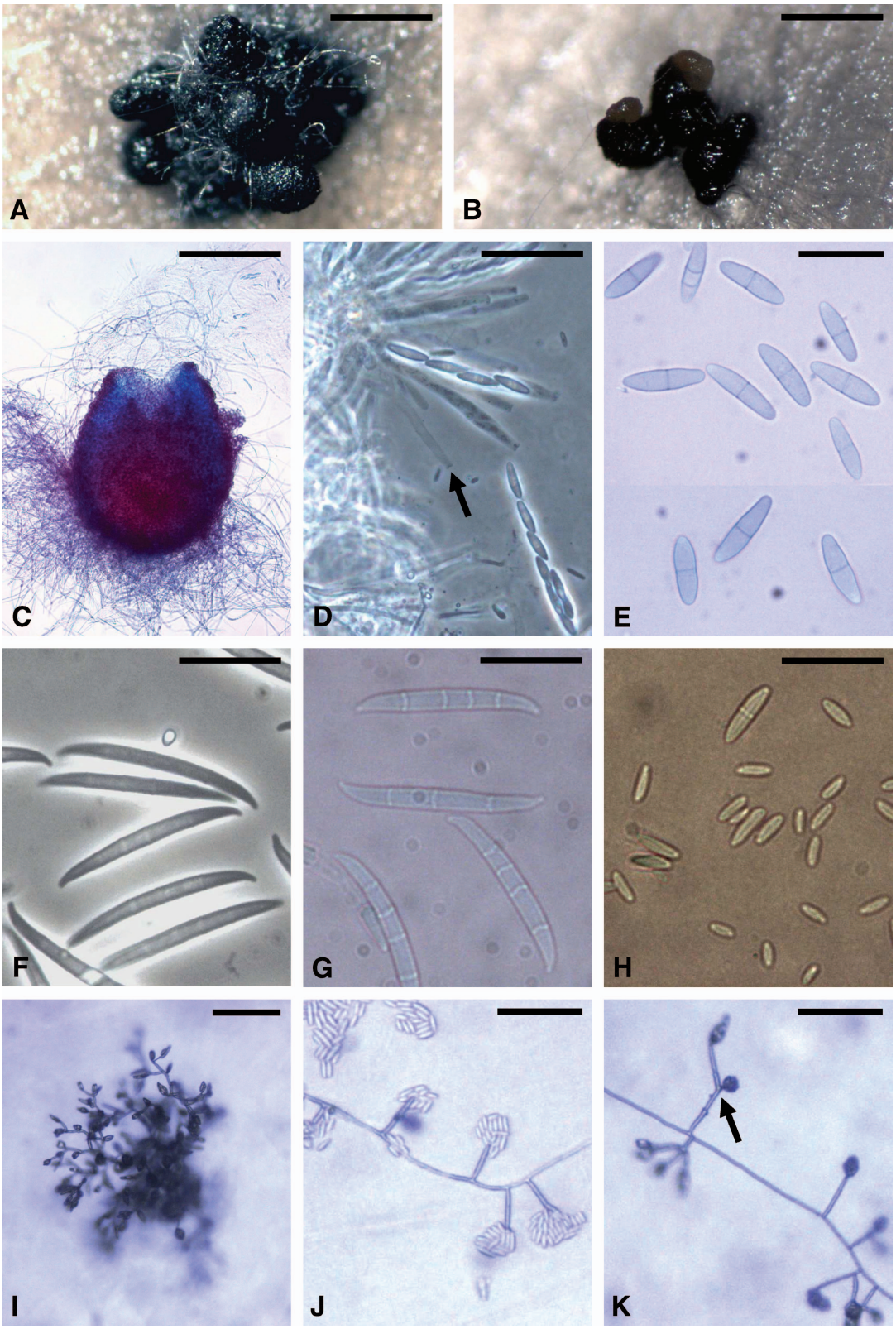

FIG. 3. *Fusarium temperatum* teleomorph from cross of strains MUCL 52463 × MUCL 52438. A. Aggregated perithecia. B. Ascospore-oozing perithecia. Bar = 0.5 mm. C. Longitudinal view of perithecium seated on a stromatic base. Bar = 0.2 mm. D. Asci cylindrical, apex with a shallow, refractive ring (arrow). Bar = 50 µm. E. One- or two-septate ascospores slightly constricted at the septum. Bar = 20 µm. *Fusarium temperatum* anamorph from strain MUCL 52463. F–G. Falcate, mostly four-septate

(GenBank number AF160301). All β-tubulin gene sequences of the *F. temperatum* strains (509 bp), including NRRL 25622 (GenBank number AF160317) were identical.

The combined β-tubulin and *EF-1α* dataset from the representative strains in this study and the sequences available from GenBank genes consisted of 1184 aligned nucleotide positions. Bayesian analysis resulted in a posterior probability distribution containing 2000 samples per analysis. The initial 1521 samples were discarded, and a majority rule consensus tree of the remaining combined samples was produced (FIG. 2). Equally weighted parsimony analysis of the 207 parsimony informative characters resulted in 18 most parsimonious trees of 745 steps. The CI and the RI for the trees generated were respectively 0.681 and 0.826. The majority rule consensus of these trees produced a tree of topology that showed no strongly supported conflicts with that produced by Bayesian analysis.

In all trees and in the majority rule consensus tree all strains of *F. temperatum* formed a strongly supported monophyletic clade (Bayesian posterior probability [PP] = 1, bootstrap [B] = 100). It is worthwhile noting that bootstrap supports obtained for *F. temperatum* monophyly when analyzing *EF-1α* and β-tubulin separately were respectively 95 and 56. In combined analyses strains of *F. subglutinans* were placed in a distinct well supported clade (PP = 1, B = 100). The overall tree topology was similar to those presented for the GFSC (O'Donnell et al. 1998a, Kvas et al. 2009), in which strain NRRL 25622 was placed in the American clade. Also the *F. circinatum* cluster was inserted between the *F. temperatum* and the *F. subglutinans* ones in a poorly supported cluster (PP = 0.75, B = 14) as observed by Lima et al. (2009).

Interspecies and intraspecies compatibility.—The *MAT1-1*:*MAT1-2* ratio among the 31 *F. temperatum* strains was 17:14 and was not significantly different from 1:1 with Chi-square statistics (TABLE I). Five *MAT1-1* strains (MUCL 52439, MUCL 52445, MUCL 52452, MUCL 52463 and MUCL 52464) and five *MAT1-2* strains (MUCL 52438, MUCL 52443, MUCL 52447, MUCL 52451 and MUCL 52462) were selected for fertility crosses from the inferred AFLP subclusters.

In an initial set of crosses against known tester isolates representing known, phylogenetically distinct species all 10 strains of *F. temperatum* were infertile when crossed with *F. subglutinans*, *F. circinatum* and *F. konzum* tester strains belonging to the American clade, except in one replicate where MUCL 52463 produced a single fertile perithecium among 150 sterile perithecia with one of the *F. subglutinans* tester strains. Internal positive controls of *F. verticillioides* tester strains were fertile.

In a second set of crosses among the 10 strains of *F. temperatum* as well as NRRL 25622 (*MAT1-2*) in all possible compatible pairs all 11 strains of *F. temperatum* were male fertile. Five strains (MUCL 52438, MUCL 52439, MUCL 52463, MUCL 52464 and NRRL 25622) showed female fertility. Nevertheless MUCL 52439 produced fertile perithecia with MUCL 52447 only. MUCL 52463 and MUCL 52438 were selected respectively as reliable female fertile tester strains of *MAT1-1* and *MAT1-2*, based on these intraspecific crosses.

TAXONOMY

Fusarium temperatum J. Scauflaire et F. Munaut, sp. nov. FIG. 3A–K

MycoBank MB518089

Coloniae in agaro PDA 4–6 mm per diem crescentes apud 25 C, post dies septem 53–75 mm diam. Mycelium aerium gossypinum, primo albidum deinde pallide roseum, rare in medio substrato tincto violaceum. Color in parte aversa pallide roseo-aurantiacus. Odor non perceptibilis. Sclerotium nulla. Chlamydosporae absentiae. Anamorphosis: Sporodochia rare in agaro SNA, pallida aurantiaca in agaro PDA. Macroconidia hyalina, 3–6 septata, plerumque 4 septata, falcata, cellula basali pediformi preadita, cellula apicali rostrata et curvata, 22–50 × 2–4 μm. Microconidia hyalina, 0–2 septata in capitulis falsis, producentes in monophialides et polyphialides, hyalina, 0 septata ellipsoidea vel ovalia et obovoidea (3–13 × 2–4 μm), magis septata fusiformia (11–23 × 3–5 μm). Microconidia numquam in catenis. Teleomorphosis: Perithecia ovoidea vel obpyriformia, superficialia, livida, 215–405 μm alta × 170–310 μm lata. Asci cylindrici, 80–100 μm alta × 6–7 μm lata, octospori, apice annulo refractivo non alto proviso. Ascosporae exudatae in cirrhis, laeves, hyalinae, ellipsoidae vel ovaliae, 1–2 septatae, plerumque 1 septatae et ad septum leviter constrictae, 13–22 × 4.5–6 μm.

Holotype. MUCL 52463-H. BELGIUM. BRABANT WALLON: Chastre, isolated from *Zea mays*, Sep 2007, dried culture. Ex holotype culture: MUCL 52463.

←

macroconidia. Bar = 20 μm. H. Ellipsoidal to oval, obovoid unseptate microconidia and fusiform one-septate microconidia. Bar = 20 μm. I. Aerial mycelium with erect, branched, polyphialidic conidiophores producing conidia either singly or in false heads. Bar = 0.1 mm. J–K. Aerial mycelium with monophialides and intercalary phialides (arrow) producing conidia in false heads. Bar = 50 μm.

Epitype. MUCL 53011-H. Dried culture with perithecia from the cross MUCL 52463 (EX HOLOTYPE) × MUCL 52438.

Colonies on PDA growing 4–6 (mean = 4.7) mm d^{-1} at 25 C in the dark, attaining 53–74 (mean = 66) mm diam after 7 d. Aerial mycelium cottony, initially white, becoming pinkish white, rarely tinged violet in the center by the substrate. Pigmentation in reverse slightly pinkish orange. Odor not perceptible. No sclerotia observed. No chlamydospores observed.

Anamorph. Sporodochia pale orange on PDA, colorless and rare on SNA. Typically macroconidia hyaline, 3–6 septa, mostly 4-septate, falcate, with a beaked curved apical cell and a footlike basal cell, 22–50 (mean = 38) µm long, 2–4 (mean = 3.3) µm wide. Conidiophores of the aerial mycelium erect, branched, terminating in 1–3 phialides. Microconidia produced either singly or in false heads on cylindrical monophialides, intercalary phialides and polyphialides, phialides up to 26 µm long and 4 µm wide. Microconidia abundant, hyaline, 0–2 septa; ellipsoidal to oval, obovoid when unseptate, 3–13 (mean = 8.2) µm long, 2–4 (mean = 2.7) wide; fusiform when 1–2 septa, 11–23 (mean = 17) µm long, 3–5 (mean = 3.9) µm wide. Microconidia not produced in chains.

Teleomorph. Perithecia ovoid to obpyriform, superficial, mostly aggregated in a small group, seated on a stroma base, and slightly warty; 215–405 (mean = 327) µm high, 170–310 (mean = 238) µm wide; dark purple in 3% KOH, turning red in lactic acid solution. Asci cylindrical, eight-spored, 80–100 (mean = 92) µm long, 6–7 (mean = 6.7) µm wide, apex with a shallow, refractive ring. Ascospores exuded in a cirrhus, smooth, hyaline, ellipsoidal to oval, 1–2 septa, mostly 1-septate (85%), slightly constricted at the septum, 13–22 (mean = 17.5) µm long, 4.5–6 (mean = 5.2) µm wide. Heterothallic species.

Etymology. The epithet *temperatum* refers to the fact that most of the isolates of this species were collected in moderate to cool and wet temperate regions.

Distribution. South Africa and Belgium. Previously studies indicated that several strains of *Fusarium* isolated worldwide were conspecific to NRRL 25622, described herein as *F. temperatum*, that suggests by extension that they also belong to *F. temperatum*. These strains also were isolated from maize in USA (Mule et al. 2004, Munkvold 2009), in Europe (Moretti et al. 2008), in cool temperate highlands of Guatemala (Torres et al. 2007) and in wet temperate regions of Mexico (Steenkamp et al. 2002).

Isolates examined. BELGIUM. BRABANT WALLON: Chastre. Isolated from *Zea mays*, MUCL 52463, *MAT1-1* (EX HOLOTYPE); BELGIUM. HAINAUT: Buissenal. Isolated from *Zea mays*, MUCL 52438, *MAT1-2*; BELGIUM. HAINAUT: Buissenal. Isolated from *Zea mays*, MUCL 52439, *MAT1-1*; BELGIUM. BRABANT WALLON: Louvain-la-Neuve. Isolated from *Zea mays*, MUCL 52443, *MAT1-2*; BELGIUM. BRABANT WALLON: Louvain-la-Neuve. Isolated from *Zea mays*, MUCL 52445, *MAT1-1*; BELGIUM. HAINAUT: Ath. Isolated from *Zea mays*, MUCL 52447, *MAT1-2*; BELGIUM. BRABANT WALLON: Louvain-la-Neuve. Isolated from *Zea mays*, MUCL 52451, *MAT1-2*; BELGIUM. BRABANT WALLON: Louvain-la-Neuve. Isolated from *Zea mays*, MUCL 52452, *MAT1-1*; BELGIUM. BRABANT WALLON: Chastre. Isolated from *Zea mays*, MUCL 52462, *MAT1-2*; BELGIUM. HAINAUT: Ath. Isolated from *Zea mays*, MUCL 52464, *MAT1-1*; SOUTH AFRICA. EASTERN CAPE: Isolated from *Zea mays*, NRRL 25622 = MRC 1077 = MUCL 51714, *MAT1-2*.

DISCUSSION

We examined the taxonomical status of *Fusarium* strains isolated from maize in Belgium that were closely related to *F. subglutinans* and to strain NRRL 25622 and described them as a new species, *Fusarium temperatum*. We used a polyphasic approach based on MSR, PSR and BSR, a strategy proposed by Taylor et al. (2000) and gained further support from AFLPs.

F. temperatum and *F. subglutinans* morphologically were similar in that they both produced conidia on monophialides and polyphialides in false heads on the aerial mycelium. On the other hand *F. temperatum* can be differentiated from *F. subglutinans* on the basis of macroconidial characteristics. The macroconidia of *F. temperatum* are mostly four-septate with a basal cell that is distinctly foot-shaped, whereas those of *F. subglutinans* were usually three-septate with a relatively poorly developed basal cell (Leslie and Summerell 2006). Nevertheless these differences are not sufficiently robust to consider them key characteristics for routine identification. We chose not to describe formally the teleomorph of *F. temperatum*, in anticipation of changes in the International Code of Botanical Nomenclature that would remove the requirement for describing both stages. We see no purpose in adding another name for this fungus to the scientific literature (Hawksworth 2009), particularly since the sexual characters associated with *F. temperatum* do not distinguish it from other species in the GFSC, and perithecia are likely to be observed only when induced in the laboratory.

The AFLP analysis clustered *F. temperatum* and *F. subglutinans* isolates into two sister groups, with a 51% Dice similarity coefficient and high bootstrap supports. This percentage is within the range of those observed among species in the GFSC or among some of the phylogenetic species represented by *F.*

graminearum and its close relatives (Zeller et al. 2003, Leslie and Summerell 2006). The genetic similarity between all *F. temperatum* pairs was 74–100%, which is consistent with descriptions of isolates belonging to the same species within the GFSC (Leslie and Summerell 2006).

Concerning PSR, all strains of *F. temperatum* formed a strongly supported monophyletic clade (PP = 1; bootstrap [B] = 100). The overall tree topology was similar to those presented for the GFSC by O'Donnell et al. (1998a). The South African isolate of *F. temperatum* does not appear to be divergent in comparison to the *F. temperatum* isolates from Belgium, suggesting that these populations might not be genetically distinct.

The interspecies and intraspecies mating compatibility assays of our study also confirmed that *F. temperatum* represents a new biological species within the American clade of the GFSC. Indeed all *F. temperatum* were infertile when crossed with the *F. circinatum* and the *F. konzum* tester strains. In one replicate MUCL 52463 produced one fertile perithecium with a *F. subglutinans* tester strain among 150 sterile perithecia. Although one single fertile perithecium cannot be considered a significant indication of interfertility, similar examples between different biological species of the GFSC already have been described (Leslie et al. 2004). Sexual compatibility between the previously described groups 1 and 2 within *F. subglutinans* was not clear-cut (Steenkamp et al. 2002). The relatively high percentage of female fertile strains and the mating-type ratio observed (*MAT1-1*:*MAT1-2* = 17:13) suggested that sexual reproduction might be common in *F. temperatum* (Leslie and Klein, 1996), and this inference is supported by the genetic diversity observed in the Belgian *F. temperatum* strains.

Of note, preliminary pathogenicity results confirm the ability of *F. temperatum* to cause stalk rot and seedling malformation at a virulence similar to *F. subglutinans* (unpubl data). The *F. temperatum*:*F. subglutinans* ratio was high in Belgian fields (276:9), suggesting that *F. temperatum* apparently competes with *F. subglutinans*. This result supported the climatic hypothesis of Moretti et al. (2008), who observed *F. subglutinans* group 2 strains in warmer and drier regions than the strains belonging to group 1. Whether this group 1 encompasses our novel species, *F. temperatum*, or contains some other yet undescribed species was not the purpose of this paper. However our results strongly suggested that *F. subglutinans* group 1 and *F. temperatum* represent highly similar evolutionary entities, if not the same thing. To confirm this hypothesis an extensive polyphasic study on strains from different origins should be conducted.

Culture extracts of *F. temperatum* NRRL 25622 were reported as producing moniliformin (Sewram et al. 1999). Furthermore strains of group 1 were shown to produce beauvericin that is not produced by *F. subglutinans* strains (Moretti et al. 2008). Experiments therefore are under way to elucidate the mycotoxin potential in *F. temperatum*.

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