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Genetic diversity and mycotoxin production of *Fusarium lactis* species complex isolates from sweet pepper

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Abstract

An internal fruit rot disease of sweet peppers was first detected in Belgium in 2003. Research conducted mostly in Canada indicates that this disease is primarily caused by *Fusarium lactis* Pirotta. Ninety-eight *Fusarium* isolates obtained from diseased sweet peppers from Belgium, as well as from other countries (Canada, the Netherlands and the United Kingdom) were identified by sequencing the translation elongation factor 1α (EF). Of these 98 isolates, 13 were identified as *F. oxysporum* Schltdl., nine as *F. proliferatum* (Matsush.) Nirenberg and two belonged to clade 3 of the *F. solani* species complex. Of the 74 remaining isolates, the EF sequence showed 97 to 98% similarity to *F. lactis*. Of these isolates, the β-tubulin (TUB), calmodulin (CAM) and the second largest subunit of RNA polymerase II (*RPB2*) genes were also sequenced. Analysis of the combined sequences revealed that the 74 isolates share nine combined sequences that correspond to nine multilocus sequence types (STs), while the *F. lactis* neotype strain and one other strain, both isolated from figs, form a separate ST. Together, these 10 STs represent a monophyletic *F. lactis* species complex (FLASC).

An unusually high level of genetic diversity was observed between (groups of) these STs. Two of them (ST5 and ST6) fulfilled the criteria for species recognition based on genealogical exclusivity and together represent a new monophyletic species lineage (FLASC-1). The seven other STs, together with the *F. lactis* neotype ST, form a paraphyletic species lineage in the African clade of the *Gibberella fujikuroi* species complex (GFSC). From each of the 10 STs, the mycotoxin production was assessed using a multi-mycotoxin liquid chromatography mass spectrometry method. Out of the 27 analyzed mycotoxins, beauvericin and fumonisins were detected in sweet pepper tissue and in maize kernels. The 10 STs clearly differed in the amount of mycotoxin produced, but there was only limited congruence between the production profile and the phylogenetic analysis. Furthermore, the morphological characterization (based on mycelial growth rate and the length of macroconidia) showed distinct differences between the 10 STs, but again there was limited congruence with the phylogenetic results.

In conclusion, the data presented in this study demonstrate that 75% of the isolates obtained from sweet pepper with internal fruit rot belong to a *F. lactis* species complex (FLASC), including a new FLASC-1 monophyletic species, and that the members of this complex display great genetic and phenotypic diversity.

Keywords

beauvericin, *Capsicum annuum*, fumonisin, *Gibberella fujikuroi,* internal fruit rot

Abbreviations

FLASC: *Fusarium lactis* species complex

1. Introduction

Sweet pepper or bell pepper (*Capsicum annuum* L.) is one of the five most important vegetable crops in most European and North American countries. These fruits are often grown in glasshouses in high-value production systems. During the last 10 to 15 years, a new disease appeared that affects the internal parts of the pepper fruit (Yang et al., 2009). In an early stage, fungal mycelium grows on the seeds or on the internal wall of the fruit. At a later stage, sunken lesions become visible on the outside of the fruit. In many cases, these external symptoms only become visible after the peppers have entered the trade circuit. Despite disease expression in the later stages of fruit development and even post harvest, the initial introduction takes place at the flowering stage via the infection of the stigma and growth through the style into the ovary (Yang et al., 2010). Fungal spores may reach the flowers via the air or via insects such as pollinating bees or bumblebees (Kharbanda et al., 2006). In the early 1990's, a fruit rot of pepper was reported in Canada and the UK (Fletcher, 1994; Jarvis et al., 1994). In both cases, *Fusarium solani* was identified as the causal agent, but in contrast to internal fruit rot, that fungus infected via external wounds of stems and pepper fruit. Internal fruit rot of sweet pepper was reported more recently, initially in the Netherlands and Canada. Between 1998 and 2002, eighteen samples of sweet pepper internal fruit rot were collected in the Netherlands. From these samples, *F. proliferatum* (11.1%), *F. solani* (11.1%), *F. oxysporum* (16.7%) and an unknown *Fusarium* spp. related to *F. lactis* (61.1%) were isolated (Hubert et al., 2003). In British Columbia, Canada, *F. subglutinans* (Wollenw. & Reinking) P.E. Nelson, Toussoun & Marasas was reported as being the causal agent of internal fruit rot of pepper (Mathur and Utkhede, 2004; Utkhede and Mathur, 2004). However, these isolates were re-identified as *F. lactis* by Yang et al. (2009). In Alberta, Canada, the disease was caused primarily by *F. lactis* (57.1%), although *F. solani* (32.1%), *F. proliferatum* (5.4%) and *F. oxysporum* (5.4%) were also recovered from some infected fruits (Yang et al., 2009). Based on these survey results and the completion of Koch's postulates by Yang et al. (2009), *F. lactis* is considered to be the most important causal agent of internal fruit rot, although other *Fusarium* species can also cause the disease. *F. lactis* is a member of the *Gibberella fujikuroi* species complex (GFSC). This complex has been intensively studied, both morphologically and molecularly (Kvas et al., 2009; Nirenberg and O'Donnell, 1998; O'Donnell et al., 1998). In the past few years, various new species of the GFSC have been described (Geiser et al., 2005; Nirenberg and O'Donnell, 1998; Nirenberg et al., 1998; Scauflaire et al., 2011; Van Hove et al., 2011; Zeller et al., 2003). The availability of molecular techniques, including the sequencing of specific

genomic regions, has contributed greatly to these discoveries (O'Donnell et al., 1998; O'Donnell et al., 2000b). The translation elongation factor 1α (EF) gene has a high phylogenetic resolution, especially in the GFSC, and is therefore one of the primary genes in phylogenetic studies in this group (Geiser et al., 2004; O'Donnell et al., 2000b). Apart from EF, several other genes containing intron-rich portions have been used to identify fungi and *Fusarium* in particular (O'Donnell et al., 2000b). These include β-tubulin (TUB), calmodulin (CAM) (Carbone and Kohn, 1999) and the second largest subunit of RNA polymerase II (*RPB2*) (Matheny, 2005; O'Donnell et al., 2007).

Members of the GFSC are known to produce several mycotoxins, such as fumonisins (FB_1, FB_2, FB_3), beauvericin (BEA), moniliformin and fusaric acid (Desjardins and Proctor, 1999; Fotso et al., 2002; Moretti et al., 2007; Rheeder et al., 2002). Since mycotoxins can cause severe diseases (Bennett and Klich, 2003; Desjardins and Proctor, 1999; Qiaomei et al., 2006), it is important to assess the presence and concentration of mycotoxins in food and feed. Fumonisins B (FBs) have been studied in detail (Alexander et al., 2009; Leslie et al., 1996; Leslie et al., 1992; Proctor et al., 2003; Proctor et al., 1999). They are produced mainly by *F. verticillioides* (Sacc.) Nirenberg and *F. proliferatum,* and primarily interfere with the sphingolipid metabolism (Richard, 2007). In the European Union, threshold levels of fumonisins range from 200 μg/kg in processed food for children and babies containing maize to 4000 μg/kg in unprocessed maize (European Commission, 2007). Recommended maximum levels for fumonisins in products intended for animal feed vary between 5 mg/kg and 60 mg/kg. Few studies exist on other mycotoxins such as beauvericin (Jestoi, 2008; Moretti et al., 2008; Yli-Mattila, 2010). Beauvericin is an insecticidal compound, isolated from *Beauveria bassiana* (Bals.-Criv.) Vuill., which is also produced by many *Fusarium* species belonging to different *Fusarium* species complexes (Logrieco et al., 1998) including the GFSC (Moretti et al., 2007). Toxicity of beauvericin towards animals and plants appears to be low to moderate (Desjardins and Proctor, 2007).

This paper describes the detailed characterization of European *Fusarium* isolates from sweet pepper, specifically the isolates closely related to *F. lactis*, based on morphological characterization, mating type determination and phylogenetic analyses of individual and combined CAM, EF, *RPB2* and TUB sequences. Additionally, mycotoxin production by representative isolates was determined in both pepper and maize.

2. Materials and methods

2.1. Fungal isolates

From the 100 *Fusarium* isolates included in this study, 82 Belgian *Fusarium* isolates were collected from sweet peppers with internal fruit rot obtained between 2005 and 2007. The peppers were acquired from commercial growers via vegetable auctions, trials at Belgian extension research centers (Hoogstraten Research Center (PCH), Belgium; Sint-Katelijne-Waver Research Station for Vegetable Production, Belgium (PSKW)), or a Belgian grocery store. Sixteen non-Belgian *Fusarium* isolates from sweet peppers were obtained from the Netherlands (9), the United Kingdom (6) and Canada (1). Two *F. lactis* reference isolates from figs (*Ficus carica* L.), including the *F. lactis* neotype strain (NRRL 25200=MUCL 51854), were obtained from the Agri-Food Toxigenic Fungi Culture Collection of the Institute of Sciences of Food Production, Bari, Italy (ITEM) (Table 1).

The fungi were isolated from sweet peppers as follows: either 1) seeds or pieces from the internal walls of the fruit were surface- sterilized via immersion in 1% NaOCl for 1 min, washed in sterile water and plated onto potato dextrose agar (PDA; Formedium, United Kingdom), or 2) mycelium was removed with a sterile needle and placed directly onto PDA. Plates were incubated at 20°C in the dark until colonies developed. From each isolate a single spore culture was prepared and used for the morphological and molecular characterization. The 74 *F. lactis* species complex (FLASC) isolates, the two *F. lactis* reference isolates, and one *F. proliferatum* isolate were deposited in the Mycothèque de l'Université catholique de Louvain (MUCL) collection (Table 1). The *F. proliferatum* strain (MUCL 53013) isolated from a sweet pepper with internal fruit rot was used as a positive control for BEA and FB analyses.

2.2. DNA extraction, PCR and sequencing

Isolates were grown on potato dextrose agar (PDA; Formedium, United Kingdom) for 3-4 weeks at 20°C in the dark. Two ml of deionized water was added to the plates and spores were released from the mycelium using a spatula. A portion of the suspension (1.2 ml) was placed in a microcentrifuge tube and centrifuged for 5 minutes at 20000 x g. The supernatant was removed and the spores were frozen in liquid nitrogen and crushed with a micropestle. This suspension was subjected to DNA extraction using the Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany).

Polymerase chain reaction (PCR) was performed with FastStart *Taq* DNA polymerase (Roche, Basel, Switzerland) in an Applied Biosystems Geneamp® PCR System 9700 (Applied Biosystems, Carlsbad, CA) using

primer pairs EF1H/EF2T for the partial EF gene (O'Donnell et al., 2000a); T1/T2 for the partial TUB gene (O'Donnell and Cigelnik, 1997); CAL228F/CAL737R for the partial CAM gene (Carbone and Kohn, 1999); and *RPB2*-1F/*RPB2*-1R for the partial *RBP2* gene (Van Hove et al., 2011). PCR reaction mixtures (total volume of 50 µL) contained 2 µL DNA (10 ng/µL) in a 1 × PCR buffer, 2.5 mM $MgCl_2$ (Fermentas GmbH, St Leon-Rot, Germany), 0.25 mM concentrations of each dNTP, 0.1 µM concentrations of each primer, and 1U FastStart *Taq* DNA polymerase. For the amplification of EF, TUB and CAM, the PCR program consisted of an initial denaturation step at 94 C for 3 min followed by 35 cycles of 30 s at 94 C, 30 s at 55 C and 1 min at 72 C. A final extension step was performed at 72 C for 5 min. The *RPB2* gene was amplified by an initial denaturation of 3 min at 95 C, followed by 39 cycles of 1 min at 94 C, 30 s at 60 C and 2 min at 72 C. After the last cycle an extension step was performed at 72 C for 10 min. PCR products were subjected to agarose gel electrophoresis. Fragments were excised and extracted from the gel using the Nucleospin® Extract II kit (Macherey-Nagel, Düren, Germany). Purified PCR products were sequenced by Macrogen Inc., Korea using the same PCR primers as for the initial amplification. To sequence the *RPB2* gene fragments, two additional primers were used: *RPB2*-2F and *RPB2*-2R (Van Hove et al., 2011). Automatic sequence assignment of the electropherograms was checked visually and contigs were assembled with Sequencher 4.0.5 (Gene Codes, Ann Arbor, MI). Representative sequences derived in this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) (FR870279-FR870326) (Table 2).

2.3. Mating type determination

The mating type of all isolates was determined by analyzing the *MAT* genes using PCR with primer pairs Gxp8/Gxp13 (Lepoint, 2006; Van Hove et al., 2011) for *MAT1-1* or GFmat2d (Steenkamp et al., 2000)/Gxp21 (Lepoint, 2006; Van Hove et al., 2011) for *MAT1-2*. PCR conditions were identical to those described above, with the following exceptions: an initial denaturation step at 94 C for 5 minutes, 30 cycles of 1 min at 94 C, 30 s at 60 C and 1 min at 72 C, followed by a final extension for 10 minutes at 72 C.

2.4. Phylogeny

In addition to the CAM, EF, *RPB2* and TUB partial gene sequences obtained in this study (Table 2), corresponding sequences for other species of the GFSC were derived from O'Donnell et al. (1998; 2000b; 2007)

and Van Hove et al. (2011), and for the *Fusarium* sp. isolates NRRL 31629 and NRRL 31630, from the *Fusarium*-ID database (http://isolate.fusariumdb.org). *RPB2* sequences from the *Fusarium* sp. isolates NRRL 31629 and NRRL 31630 were kindly provided by Kerry O'Donnell, United States Department of Agriculture, Peoria, IL. The sequences were aligned initially using CLUSTAL X software (Thompson et al., 1997) and then manually edited. The multiple sequence alignment was deposited in TreeBASE and is available at http://purl.org/phylo/treebase/phylows/study/TB2:S11563. Phylogenetic analyses were performed using PAUP* version 4.0b10 (Swofford, 2003) on the individual and combined datasets, using the heuristic search option with 1000 random addition sequences with MULPARS on and tree bisection-reconnection (TBR) branch swapping. Phylogenetically informative indels were coded as a single event (i.e., fifth state). The GFSC gene trees were rooted by the outgroup method using sequences of a putative sister group, *F. oxysporum* species complex isolates NRRL 22902 and NRRL 20433, as described in O'Donnell (1998). The FLASC gene trees were rooted using sequences of the two closest species, *F. denticulatum* isolate NRRL 25302 and *F. pseudocircinatum* isolate NRRL 22946. Stability of clades was assessed by 1000 parsimony bootstrap replications, using heuristic searches with 1000 replicates of random sequence addition implemented with PAUP*.

2.5. Growth rate and morphology

In the growth rate and morphological studies, two representative isolates of each of the FLASC STs were used, except for ST8 and ST9, which were represented by a single isolate (Tables 1 and 3). Growth rate was determined from cultures grown on PDA at 25°C in the dark. For microscopic analysis of microconidia and phialides, cultures were grown on Synthetischer Nährstoffarmer Agar (SNA) at 20°C in the dark (Leslie and Summerell, 2006). For microscopic analysis of macroconidia, cultures were grown on banana leaf agar (BLA) at 24°C under continuous fluorescent light. BLA was prepared by placing autoclaved banana leaf pieces on 2% water agar. Cultures on SNA and BLA were examined microscopically *in situ* and as a slide preparation at 400x magnification using an Olympus BX50 microscope (Tokyo, Japan) and Leica DFC280 camera image capture system (Wetzlar, Germany). The length of a minimum of 30 arbitrarily chosen micro- and macroconidia per isolate was determined using Leica IM500 imaging software (Wetzlar, Germany). The number of septa of each macroconidium was counted. Data were statistically analyzed using ANOVA and Bonferroni posthoc tests (at the isolate level) or Tukey's multiple range tests (at the ST level) with Statistica 9.0 (Statsoft, Tulsa, USA).

2.6. Multi-mycotoxin analysis

Commercial red sweet peppers were inoculated with one of the following: *F. proliferatum* isolate, two *F. lactis* reference strains and 17 FLASC isolates from sweet pepper representing ST1-ST9 (Tables 1 and 4). The peppers were inoculated and analyzed according to the method described by Monbaliu et al. (2009), except that the inoculated peppers were incubated at 20°C for 2 weeks instead of 1 week and that three additional mycotoxins were analyzed. In total, 27 mycotoxins were included in the multi-mycotoxin analyses: aflatoxins (aflatoxin B_1, aflatoxin B_2, aflatoxin G_1 and aflatoxin G_2), *Alternaria* toxins (altenuene, alternariol and alternariol methyl ether), BEA, enniatin B, fumigaclavin, FB_1, FB_2, FB_3, ochratoxin A, paxillin, roquefortin C, sterigmatocystin, trichothecenes (3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, deoxynivalenol, diacetoxyscirpenol, fusarenon-X, HT-2 toxin, neosolaniol, nivalenol, and T-2 toxin) and zearalenone. To investigate the mycotoxin production in maize, sterilized maize kernels were inoculated with the above isolates in triplicate and analyzed for the same spectrum of mycotoxins. Ten ml of water was added to 20 g of kernels in 250 ml Erlenmeyer flasks, autoclaved twice, and inoculated with 2 x 10^4 conidiospores in 10 ml 0.1% Tween-20. After blending and homogenization of the maize samples, two grams were added to 10 mL ethyl acetate/formic acid (99/1, v/v), agitated using an end-over-end tumbler for 60 min and centrifuged for 15 min at 3300 g. The supernatant was transferred into a test tube and evaporated. The residue was dissolved in 250 µL mobile phase for injection (water/methanol, 60/40, v/v containing 0.3% formic acid) and centrifuged for 10 min at 3300 g. One hundred seventy-five µL was transferred into a Millipore ultrafree MC centrifugal device of 0.22 µm (Bedford, MA) and centrifuged for 10 min at 14000 g. Ten µL was injected for the ultra high performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) analysis. The detailed analytical conditions were described by Monbaliu et al. (2010b).

3. Results

3.1. Molecular characterization

Initially, the EF gene was partially sequenced to help identify the 98 *Fusarium* isolates (Tables 1 and 2). For 24 isolates (24.5%) the EF sequence was identical to that of a *Fusarium* species other than *F. lactis*. This included *F. oxysporum* (13.3%), *F. proliferatum* (9.2%) and *F. solani* species complex clade 3 (2.0%). For the other 74

isolates (75.5%), six EF sequences (designated EF1-6) were obtained (Table 2). The EF6 sequence, which corresponds to the MUCL 51854 (=NRRL 25200) and MUCL 51855 isolates, was identical to the EF sequence of the *F. lactis* neotype strain (NRRL 25200) available in Genbank and the *Fusarium*-ID database. The other five EF sequences (EF1-5) were more closely related to the *F. lactis* neotype sequence (97-98% similarity) than to EF sequences of other *Fusarium* species in Genbank. Interestingly, EF1-5 had the highest similarity (98-100%) with the *Fusarium* isolates NRRL 31629, NRRL 31630, NRRL 31632, and NRRL 31633 from the *Fusarium*-ID database. All of those isolates originated from sweet peppers collected in South Africa. Based on this, we included the sequence data from isolates NRRL 31629 and NRRL 31630 in the present study. The NRRL 31629 EF sequence represented a seventh distinct sequence (EF7), and the EF sequence of NRRL 31630 was identical to EF1 (Table 2).

The 74 FLASC isolates from sweet pepper and the two *F. lactis* reference isolates were further characterized by sequencing and phylogenetic analysis of specific parts of their CAM, *RPB2* and TUB genes. Together with corresponding sequence data from isolates NRRL 31629 and NRRL 31630, this resulted in seven CAM sequences (CAM1 through 7), seven *RPB2* sequences (*RPB2*-A through G), and seven TUB sequences (TUB1 through 7), (Tables 1 and 2). Only the TUB partial sequences from all of the 74 FLASC isolates differed from those in the *F. lactis* reference strains NRRL 25200 (=MUCL 51854) and NRRL 25338 (=MUCL 51855), while at least some isolates shared identical CAM, EF and *RPB2* sequences with these reference strains. When the CAM, EF, *RPB2* and TUB sequences were combined, this resulted into twelve multilocus sequence types (STs) with unique combined sequences, which corresponded to nine FLASC STs (ST1 to ST9) among the 74 sweet pepper isolates, two South African isolates NRRL 31629 (ST10) and NRRL 31630 (ST11), and one *F. lactis sensu stricto* ST (ST12) (Tables 1 and 2). Comparison of their individual CAM, EF, *RPB2* and TUB partial sequences showed that NRRL 31629 (ST10) differed from ST1 to ST9 and ST12 for the CAM (CAM6), EF (EF7) and TUB (TUB7) partial sequences, but shared the same *RPB2* partial sequence (*RPB2*-A) with ST1. NRRL 31630 isolate (ST11) differed only in the CAM (CAM7) partial sequence while it had the same EF, *RPB2* and TUB sequences as FLASC ST1 (Table 2).

The mating type of each isolate was also determined. Each of the nine FLASC STs had a single mating type. All isolates from FLASC ST2, ST5, ST6 and ST9 and the *F. lactis sensu stricto* ST12 were *MAT1-1*, while the isolates from FLASC ST1, ST3, ST4, ST7 and ST8 were *MAT1-2* (Table 2).

Phylogenetic analyses were conducted on partial sequences of CAM, EF, *RPB2* and TUB, individually and combined. Alignment of CAM, EF, *RPB2* and TUB partial sequences from the FLASC ST1 to ST12, together with sequences from other GFSC species, included 398, 668, 1753 and 1092 nucleotide positions, respectively, totaling 3911 aligned nucleotide positions. There were 2927 constant characters, 379 parsimony-uninformative variable characters, and 605 parsimony-informative characters. Of these 605 parsimony-informative characters, 65 were from CAM, 155 from EF, 243 from *RPB2* and 142 from TUB. Maximum parsimony heuristic analysis of the combined dataset yielded 12 most parsimonious trees (MPTs) (Figure 1). When the alignment of CAM, EF, *RPB2* and TUB partial sequences was restricted to the FLASC ST1 to ST12, there were 3671 constant characters, 145 parsimony-uninformative variable characters, and 95 parsimony-informative characters. Of these 95 parsimony-informative characters, 7 were from CAM, 25 from EF, 53 from *RPB2* and 10 from TUB. Maximum parsimony heuristic analysis of the restricted combined dataset yielded three MPTs (length = 332 steps; CI = 0.747; RI = 0.682) (data not shown). The full combined analysis including GFSC species sequences (Figure 1) and the corresponding four individual gene analyses (data not shown) revealed that FLASC ST1-ST9 and both South-African isolates NRRL 31629 (ST10) and NRRL 31630 (ST11) are nested within the African clade of the GFSC and are most closely related to *F. lactis sensu stricto* ST12. Together, these 12 STs form a monophyletic FLASC clade (highlighted in grey in Figure 1), which is strongly supported by a 100% maximum parsimony bootstrap value. The four individual CAM, EF, *RPB2* and TUB MPTs are topologically concordant with strong bootstrap support of the FLASC clade with values of 95%, 85%, 77% and 98%, respectively, in the full analysis (data not shown). This clade is also highly supported in each individual CAM, EF and TUB trees with strong bootstrap support values of 96%, 100% and 99%, respectively, in the restricted analysis, but is not significantly supported (60% bootstrap value) in the *RPB2* tree (Figure 2). In the EF tree, one fully supported (100%) subclade groups FLASC ST1 to ST4 together with ST11, while another well supported (88%) subclade groups FLASC ST5 to ST7 (Figure 2). In the *RPB2* tree, two 100% supported subclades associate FLASC ST5 and ST6, on the one hand, and FLASC ST1, ST10 and ST11, on the other hand (Figure 2). In the TUB tree, only one subclade composed of FLASC ST1 to ST4, ST10 and ST11 is strongly (95%) supported (Figure 2). No significantly supported (>75%) FLASC subclade was observed in the CAM tree. The full combined analysis (Figure 1) revealed that ST7 is in a basal position within the FLASC clade while the other STs are grouped in one significantly supported subclade (80% bootstrap value). However, the four restricted individual gene tree topologies (Figure

2) revealed strong inconsistencies with respect to the basal position of the FLASC ST7 and the monophyly of the large subclade, since ST7 is strongly associated with other STs in three of the four individual analyses, *i.e.* CAM, EF and TUB. Three smaller subclades were strongly supported (bootstrap value >75%) in the combined analysis (Figure 1). Of these, only the ST5-ST6 subclade (FLASC-1) fulfills the criteria of multilocus phylogenetic speciation. The two NRRL isolates (ST10 and ST11) are closely associated with high bootstrap support in all individual gene analyses, except in the EF analysis. Similarly, although the FLASC ST1 to ST4 are closely associated in the EF and TUB analyses with significant bootstrap support (100% and 95%, respectively), as well as within the CAM analysis although with no significant support (bootstrap value < 75%), they are clearly dispersed within the *RPB2* analysis. Consequently, neither ST1 to ST4 nor ST10 and ST11 are forming, separately or together, a significantly (bootstrap value >75%) supported clade in the combined analysis.
Out of the 10 known Belgian origins (growers), six contained isolates from at least two FLASC STs and three origins contained isolates from at least three FLASC STs. When the number of isolates sampled per origin was large, as was the case with the 27 isolates of origin 2, isolates from six different FLASC STs could be detected. At this origin, only two out of six FLASC STs appeared in both 2006 and 2007, but these were represented by 17 isolates (63%).

3.2. Growth rate and morphology

Differences in growth rate among representative isolates of FLASC ST1-ST9 and the *F. lactis sensu stricto* ST12 were observed. Especially the growth rates of FLASC ST2, ST8 and ST9 isolates were significantly higher than those of ST12 isolates (Table 3). Microconidia of representative isolates of FLASC ST1-ST9 and the *F. lactis sensu stricto* ST12 were ovoid to obovoid and were formed in false heads and short- to medium-length chains. Mono- and polyphialides were observed in every isolate. The average size of the microconidia ranged from 5.8 µm (ST9) to 6.7 µm (ST8). No significant differences were observed between the STs, except for the isolates of ST8 and ST9 (Table 3). When grown on BLA, 13 of the 18 isolates tested produced macroconidia-containing sporodochia. These were all slender and only slightly curved, with a bent apical cell and a notched basal cell. Significant differences in size and number of septa of the macroconidia were observed, especially between the smaller macroconidia of FLASC ST3, ST5 and ST6 (27.3 – 38.7 µm; 2.8 -3.2 septa) and the larger ones from ST4 (53.1 – 54.1 µm; 4.6 septa). Similar differences were seen in the average number of septa (Table 3).

3.3. Multi-mycotoxin analysis

Twenty-seven mycotoxins were analyzed in sweet pepper using two *F. lactis sensu stricto* ST12 isolates and 18 isolates from sweet pepper that represent FLASC ST1 to ST9. As *F. proliferatum* is known to produce high levels of FBs, one isolate was included in the experiment as a control. Because maize kernels are a good substrate for mycotoxin production, the isolates were also inoculated on sterile maize kernels. At least some of the FLASC isolates produced BEA, FB_1, FB_2 and/or FB_3 (Table 4) but none of the isolates produced any of the other 23 mycotoxins. As expected, the *F. proliferatum* isolate produced high levels of BEA, FB_1, FB_2 and FB_3 and the mycotoxin levels were considerably higher in maize than in pepper. No mycotoxins were detected in sweet pepper inoculated with *F. lactis sensu stricto* ST12 isolates, and with some or all tested isolates of FLASC ST3, ST5, ST7, ST8 and ST9. The FLASC isolates of ST1, ST2, ST4, and one isolate of ST5 and ST6 produced moderate to high levels of BEA in sweet pepper (94 to 161,000 µg/kg). The *F. proliferatum* isolate produced limited amounts of BEA in pepper. Traces of FB_1 (< LOQ), were only detected in pepper samples infected with one isolate of ST1 (MUCL 51511) and one isolate of ST2 (MUCL 51519). The latter also produced traces (< LOQ) of FB_2 and FB_3.

In general, the mycotoxin levels were considerably higher in maize than in pepper, except for BEA produced by the MUCL 52697 ST6 isolate, and for FB_2 and FB_3 production by the MUCL 51519 ST2 isolate. The *F. lactis sensu stricto* ST12 isolates produced moderate levels of BEA (434 to 4,630 µg/kg) on maize but no FB_1, FB_2 or FB_3. Low to moderate amounts of BEA (65 to 3,220 µg/kg) were produced by the isolates of ST3, two isolates of ST5 and the isolates of ST8 and ST9. Higher amounts of BEA were produced by the ST5 isolate MUCL 52693 and the ST6 isolate (57,300 and 97,300 µg/kg, respectively). For isolates of ST1, ST2 and ST4 even higher BEA amounts (289,000 to 4,280,000 µg/kg) were recorded. Regarding fumonisin detection in maize inoculated with isolates of ST1-ST4, moderate levels of FB_1 were detected (150 to 1,800 µg/kg) as compared to *F. proliferatum* (77,000 µg/kg). Only low amounts of FB_2 and FB_3 were produced in maize by two isolates of ST2 (MUCL 51516 and MUCL 51529). Similarly to the *F. lactis sensu stricto* isolates, the isolates of ST5-9, and one isolate of ST1 (MUCL 51518) did not produce fumonisins in maize.

4. Discussion

Yang et al. (2009; 2010) recently described the causal organisms and the etiology of internal fruit rot of sweet pepper, mostly based on Canadian isolates. They isolated at least four different *Fusarium* species from infected pepper fruits and stems, including *F. proliferatum*, *F. oxysporum*, *F. lactis* and *F. solani*. Of these, while being more aggressive, *F. solani* seems to be more confined to infection of the pepper stems and the external part of the fruit and is therefore not a cause of internal fruit rot in the strict sense. In their study, the majority (57.1%) of the *Fusarium* isolates causing internal fruit rot was *F. lactis*. Similar results have been obtained by Hubert et al. (2003) in the Netherlands. Our survey also revealed that the majority (75.5%) of our isolates formed a strongly supported lineage (*F. lactis* species complex; FLASC) with the *F. lactis* neotype strain (MUCL 51854=NRRL 25200), while other isolates were identified as *F. proliferatum* (9.2%), *F. oxysporum* (13.3%) and *F. solani* species complex clade 3 (2.0%). Although our study contained a limited number of isolates from the Netherlands (9) and the UK (6), isolates belonging to the FLASC were also dominant in these regions (77.8% and 50.0%, respectively). Based on morphological analysis, *F. subglutinans* was originally identified as being the causal agent of internal fruit rot of sweet pepper in British Colombia (Utkhede and Mathur, 2004). However, these isolates were later re-classified as *F. lactis* using multi-gene sequence analysis (Yang et al., 2009). The present multi-gene study confirmed that the main causal agents of internal fruit rot of sweet pepper in various European countries as well as in various Canadian provinces were FLASC isolates. These isolates form a monophyletic FLASC clade located within the African clade of the GFSC. Indeed, this clade satisfies the genealogical concordance and nondiscordance criteria (Dettman et al., 2003) as (i) the clade is present in the majority (4/4) of the single-locus genealogies; (ii) the clade is well supported in at least one single-locus genealogy, *i.e.,* bootstrap support values of 96%, 100% and 99% in CAM, EF and TUB, respectively; and (iii) the clade is not contradicted in any other single-locus genealogy at the same level of support (Figures 1 and 2). Among the 74 isolates from our survey, we could distinguish nine phylogenetic FLASC STs, which in some cases differed significantly from the *F. lactis sensu stricto* ST12, which includes the *F. lactis* neotype strain. Notably, the gene sequences of the two isolates from South Africa (NRRL 31629 and NRRL 31630) shared higher similarity with sequences from the nine FLASC STs and the *F. lactis sensu stricto* ST12 than with other *Fusarium* species of the GFSC. Consequently, these two isolates were also phylogenetically nested within the FLASC clade. The 78 isolates of the FLASC clade are represented by 12 partial CAM, EF, *RPB2* and TUB combined sequences that share relatively low pairwise similarity (97.0%). Interestingly, the Canadian isolates of *F. lactis*

had an even higher level of sequence variability, as these isolates shared only 95% sequence similarity with *F. lactis* EF, TUB and mtSSU reference sequences (Yang et al., 2009). The rather low similarity (97.0%) between the 12 FLASC combined sequences (3911 bp) obtained in our study was noticeably lower than intra- and inter-species sequence similarities observed within and between some species of the GFSC. Indeed, in the first case the lowest pairwise similarities observed between partial CAM, EF, *RPB2* and TUB combined sequences (3911 bp) from nine *F. verticillioides* and six *F. musae* haplotypes of various geographic origins were 99.6% and 99.5%, respectively. Moreover, the level of pairwise similarity between the 15 combined sequences from both species was not less than 98.9% (Van Hove et al., 2011 and personal communication). In the second case, when examining the inter-species pairwise similarities between every possible pair (435) of combined sequences from the 30 phylogenetic *Fusarium* species of the GFSC used in this study, eight pairs have a value strictly higher than 97.0%, *i.e., F. verticillioides* versus *F. musae* (97.8%), *F. anthophilum* versus *F. succisae* (97.6%), *F. bulbicola* versus *F. anthophilum* (97.4%), *F. proliferatum* versus *F. globosum* (97.4%), *F. bulbicola* versus *F. succisae* (97.3%), *F. pseudoanthophilum* versus *F. brevicatenulatum* (97.1%), *F. bactridioides* versus *F. subglutinans* (97.1%) and *F. anthophilum* versus *F. begoniae* (97.1%) (Supplementary Table 1). This higher genetic variability within the FLASC is also characterized by the presence of a FLASC subclade composed of ST5 and ST6 that differs significantly from the *F. lactis sensu stricto* subclade. This points to a new phylogenetic sister species to *F. lactis sensu stricto*. This hypothesis was confirmed as the ST5-ST6 subclade fulfills the criteria of multilocus phylogenetic speciation (Dettman et al., 2003). In contrast, our phylogenetic analysis demonstrated that, although the criteria of multilocus phylogenetic speciation at the level of the individual genes were fulfilled, the clade that consists of ST1 to ST4, ST10 and ST11 does not form a separate sister species because of the low support (bootstrap value <75%) in the combined analysis.

Notably, both mating types (*MAT1-1* and *MAT1-2*) are present within the FLASC. This indicates the possibility of sexual compatibility and ability to produce sexual fruiting structures bearing fertile progeny. However, mating experiments are needed to confirm this hypothesis. It is worthwhile to note that only one mating type (*MAT1-1*) is present in all isolates from the new phylogenetic species FLASC-1.

Phenotypic analyses were also performed to uncover the metabolic and morphological diversity within FLASC and compare it with the genetic diversity. The knowledge of secondary metabolite production by fungal plant pathogens is extremely important when evaluating the potential toxicity of infected plant products and

characterizing the isolates within the GFSC (Moretti et al., 2007). The mycotoxins produced by representative FLASC isolates from sweet pepper were determined in sweet pepper and in maize kernels using a multi-mycotoxin LC-MS/MS technique (Monbaliu et al., 2009). Out of the 27 mycotoxins analyzed, only BEA, FB_1, FB_2 and FB_3 were detected in sweet pepper tissue and in maize. In general, a similar production profile was observed for pepper and maize but the concentrations detected in maize were considerably higher, except for BEA in ST6 (isolate MUCL 52697). Based on these mycotoxin profiles, FLASC STs could be divided into two main groups, *i.e.* isolates from ST1-4 that are producing FBs while ST5-9 and ST12 are not producing FBs. Similarly, differential production of beauvericin was observed between phylogenetic within-species groups of *F. subglutinans* (Moretti et al., 2008). Very interestingly, this fumonisin production pattern is supporting the hypothesis of a ST1-4 separate lineage within the FLASC though this is not congruent with the multi-gene based phylogeny. However, more isolates per ST should be tested for mycotoxin production to improve the statistical significance of the comparison. ST1-4 showed good agreement between the individual isolates. This was in contrast with ST5, whose isolate MUCL 52693 produced high levels of BEA in pepper and in maize, while its isolates MUCL 52692 and MUCL 52694 produced small amounts of BEA, and only in maize. The *F. lactis sensu stricto* isolates included in this research produced BEA in maize. The literature shows contradictory results for *in vitro* BEA production by *F. lactis sensu stricto* isolates. Fotso et al. (2002) reported no BEA production while Moretti et al. (2007) published the *in vitro* production of 190,000 µg/kg BEA by one isolate of *F. lactis sensu stricto* from *Ficus carica*. Regarding fumonisin, our results are the first report of FB production by some FLASC isolates on maize and pepper, specifically strains from ST1-ST4. Our results are consistent with previous data indicating that FB was not produced by *F. lactis sensu stricto* (Fotso et al., 2002; Kvas et al., 2009). Monbaliu et al. (2010a) performed a migration study that showed no migration of BEA beyond the fungal affected part, while FBs did migrate to some extent into the surrounding healthy sweet pepper tissue. Our study indicates that the majority of the FLASC isolates involved in internal fruit rot in pepper produced low to moderate amounts of BEA and in some cases, very low levels of FBs (< LOQ) in pepper. Therefore, if the affected part of the pepper is removed, consumption of BEA and FBs will be negligible in most cases.

Colony colour and colony morphology of the isolates belonging to different FLASC STs differed (data not shown), but these were sometimes variable within a ST. The growth rate of ST2 was significantly higher than that of ST1, ST5, ST6, ST7 and ST12. Although not always statistically significant, the growth rate of (isolates of)

ST5 and ST6 was lower than that of fast growing isolates of ST2, ST3 and ST4, supporting designation of FLASC-1. Significant differences in size of the microconidia were only observed between ST8 and ST9 (Table 3). However, significant differences in the size of the macroconidia were present. Isolates of ST3, ST5 and ST6 produced smaller macroconidia than isolates of ST1, ST2, ST4, ST8 and ST12. The slow growth and small macroconidia of ST5 and ST6 are distinguishing characters that support FLASC-1 being a new phylogenetic species.

The large genetic and phenotypic diversity of the *Fusarium* isolates that cause internal fruit rot of sweet pepper is striking, especially given that the disease has been observed for less than 10 years in most sweet pepper growing regions. Epidemic spread between greenhouses is unlikely, given the high diversity coupled with the disease emergence in the same period in many countries and regions. Spread via infected sweet pepper seed has been hypothesized (Yang et al., 2009; Yang et al., 2010) but the diversity in fruit-rot-causing *Fusarium* species and FLASC STs does not suggest spread from a central source. For the same reason it is unlikely that the sweet pepper FLASC STs all originate directly from figs, in which *F. lactis* has been described to also cause an internal fruit rot (endosepsis) (Michailides, 2003; Nirenberg and O'Donnell, 1998). Given that *F. proliferatum*, *F. oxysporum*, and at least nine FLASC STs are able to cause the disease and that their pathogenicity is relatively low on sweet pepper (Yang et al., 2009), the interaction is not very specific. Therefore, we hypothesize that reduced resistance to these fungi in recent sweet pepper cultivars has contributed to the relatively short term and rather general emergence of the disease. Inoculation experiments on older pepper cultivars could test this hypothesis. Epidemiological studies will have to elucidate whether these fungi are common in the greenhouse environment or specifically introduced with the seed or during the growth of a sweet pepper crop. Preliminary air sampling in a tomato greenhouse led to the identification of three *F. proliferatum* isolates and one FLASC ST2 isolate (data not shown), indicating that the fungi that cause internal fruit rot of bell pepper may be generally present in the air, though possibly at low concentrations. Environmental factors may also have contributed to an increase in the disease problems. Specifically, changes in climate control in European greenhouses as a consequence of higher energy prices may allow higher relative humidity and a longer period of condensation on the flowers, which could favour infection of the stigmas.

Although this paper contributes to the understanding of this intriguing disease and the risks it may pose to consumer health, further research is necessary to better resolve the FLASC species phylogeny and to decipher

the epidemiology (additional sampling in greenhouses and in natural environments), sexual fertility and behaviour (laboratory mating experiments), genetic bases of differences in mycotoxin production patterns (BEA and FB biosynthesis gene analysis), genetic diversity (e.g. VNTR analyses) and pathogenicity (inoculation of flowers) of its causal agents.

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Tables

Table 1. Details of the 78 *Fusarium lactis* species complex isolates from sweet pepper used in this study.

Isolates used in the morphological and the mycotoxin analyses are indicated in italics and bold, respectively.

Strain #[a]	Isolate #	Multilocus sequence type #	Origin		Collection year[b]
			country	grower[b,c]	
MUCL 51511	***PSKW12***	ST1	BE	1	2007
MUCL 51512	PSKW29	ST1	BE	1	2007
MUCL 51513	PSKW31	ST1	BE	1	2007
MUCL 51514	PSKW35	ST1	BE	1	2007
MUCL 51518	**PCH09**	ST1	BE	2	2007
MUCL 51520	PCH12	ST1	BE	2	2007
MUCL 51521	VH01	ST1	BE	3	2007

MUCL 51524	***VH06***	ST1	BE	4	2007
MUCL 51526	VH08	ST1	BE	3	2007
MUCL 51528	VM04	ST1	BE	5	2007
MUCL 51530	VM09	ST1	BE	6	2007
MUCL 51531	VM10	ST1	BE	7	2007
MUCL 51532	VM11	ST1	BE	7	2007
MUCL 51533	KHK31	ST1	BE	8	2006
MUCL 51534	KHK37	ST1	BE	4	2006
MUCL 51535	KHK49	ST1	BE	9	2006
MUCL 51536	KHK53	ST1	BE	10	2006
MUCL 51537	KHK62	ST1	BE	7	2006
MUCL 51814	Fus405	ST1	UK	ND	ND
MUCL 51819	KVP01	ST1	BE	grocery	2007
MUCL 51823	PCHpaars	ST1	BE	ND	ND
MUCL 51937	PCH08	ST1	BE	2	2007
MUCL 51938	PCH10	ST1	BE	2	2007
MUCL 51939	VH02	ST1	BE	3	2007
MUCL 51940	VH03	ST1	BE	3	2007
MUCL 51941	KHK01	ST1	BE	2	2006
MUCL 51942	KHK11	ST1	BE	2	2006
MUCL 51943	KHK30	ST1	BE	2	2006
MUCL 51944	KHK40	ST1	BE	4	2006
MUCL 51945	KHK54	ST1	BE	10	2006
MUCL 51946	KHK55	ST1	BE	10	2006
MUCL 51947	KHK63	ST1	BE	7	2006
MUCL 51948	Fnyg2	ST1	BE	2	2005
MUCL 51949	62c	ST1	BE	ND	ND
MUCL 51950	Fus406	ST1	UK	ND	ND
MUCL 51951	Fus409	ST1	UK	ND	ND
MUCL 51516	**PCH01**	ST2	BE	2	2007
MUCL 51519	***PCH11***	ST2	BE	2	2007
MUCL 51529	***VM08***	ST2	BE	6	2007
MUCL 51813	672A	ST2	NL	ND	2008
MUCL 51815	KHK05	ST2	BE	2	ND
MUCL 51816	KHK13	ST2	BE	2	2006
MUCL 51817	KHK22	ST2	BE	2	2006
MUCL 51820	PCH04	ST2	BE	2	2007
MUCL 51952	PCH02	ST2	BE	2	2007
MUCL 51953	PCH03	ST2	BE	2	2007
MUCL 51954	KHK12	ST2	BE	2	2006
MUCL 51955	KHK27	ST2	BE	2	2006
MUCL 51957	PCHoranje	ST2	BE	2	ND
MUCL 51515	PSKW39	ST3	BE	1	2007

MUCL 51517	***PCH05***	ST3	BE	2	2007
MUCL 51522	**VH04**	ST3	BE	3	2007
MUCL 51525	VH07	ST3	BE	3	2007
MUCL 51527	***VM03***	ST3	BE	5	2007
MUCL 51821	PCH06	ST3	BE	2	2007
MUCL 51822	PCH13	ST3	BE	2	2007
MUCL 51824	PSKW41	ST3	BE	1	2007
MUCL 51523	***VH05***	ST4	BE	8	2007
MUCL 51818	***KHK35***	ST4	BE	8	2006
MUCL 52692	***PSKW13***	ST5	BE	1	2007
MUCL 52693	***PSKW40***	ST5	BE	1	2007
MUCL 52694	**PCH07**	ST5	BE	2	2007
MUCL 52697	***666B***	ST6	NL	ND	2008
MUCL 52799	666A	ST6	NL	ND	2008
MUCL 52804	*668A*	ST6	NL	ND	2008
MUCL 52805	668B	ST6	NL	ND	2008
MUCL 52806	667A	ST6	NL	ND	2008
MUCL 52807	667B	ST6	NL	ND	2008
MUCL 52695	***VM06***	ST7	BE	5	2007
MUCL 52800	61d	ST7	BE	ND	2006
MUCL 52801	*KHK19*	ST7	BE	2	2006
MUCL 52802	KHK20	ST7	BE	2	2006
MUCL 52696	***F2004-C***	ST8	CA	Alberta	2004
MUCL 52803	***KHK21***	ST9	BE	2	2006
NRRL 31629		ST10	ZA	ND	2001
NRRL 31630		ST11	ZA	ND	2001
[T] MUCL 51854[e]		ST12	US	ND	1994
MUCL 51855[e]		ST12	US	ND	1994

[a] MUCL, Mycothèque de l'Université catholique de Louvain, Université catholique de Louvain, Louvain-la-Neuve, Belgium; NRRL, ARS culture collection, NCAUR, Peoria, IL; [T], neotype of *F. lactis*.

[b] ND: no data

[c] Grower data was coded.

[d] NA: not applicable

[e] MUCL 51854 = CBS 411.97 = ITEM 3541 = NRRL 25200; MUCL 51855 = CBS 420.97 = ITEM 3542 = NRRL 25338 (CBS, CentraalBureau voor Schimmelcultures, Utrecht, The Netherlands; ITEM, Agri-Food Toxigenic Fungi Culture Collection, Institute of Sciences of Food Production, Bari, Italy)

Table 2. Genetic description of *Fusarium lactis* species complex multilocus sequence types (ST1 to ST12) from sweet pepper.

ST #	# of isolates	Representative isolates[a]	EF[b]		CAM[c]		*RPB2*[d]		TUB[e]		MAT[f]
			group	GenBank accession #	group	GenBank accession #	group	GenBank accession #	group	GenBank accession #	
ST1	36	MUCL 51511	1	FR870279	1	FR870291	A	FR870303	1	FR870315	2
ST2	13	MUCL 51516	1	FR870280	2	FR870292	B	FR870304	1	FR870316	1
ST3	8	MUCL 51515	1	FR870281	2	FR870293	C	FR870305	1	FR870317	2
ST4	2	MUCL 51523	2	FR870282	3	FR870294	D	FR870306	1	FR870318	2
ST5	3	MUCL 52692	3	FR870283	4	FR870295	E	FR870307	2	FR870319	1
ST6	6	MUCL 52697	3	FR870284	4	FR870296	E	FR870308	3	FR870320	1
ST7	4	MUCL 52695	3	FR870285	4	FR870297	F	FR870309	2	FR870321	2
ST8	1	MUCL 52696	4	FR870286	5	FR870298	B	FR870310	4	FR870322	2
ST9	1	MUCL 52803	5	FR870287	4	FR870299	G	FR870311	5	FR870323	1
ST10	1	NRRL 31629	7	FR870288	6	FR870300	A	FR870312	7	FR870324	ND[g]
ST11	1	NRRL 31630	1	FR870289	7	FR870301	A	FR870313	1	FR870325	ND[g]
ST12	2	[T] MUCL 51854	6	FR870290	4	FR870302	G	FR870314	6	FR870326	1

[a] MUCL, Mycothèque de l'Université catholique de Louvain, Université catholique de Louvain, Louvain-la-Neuve, Belgium; [T], neotype of *F. lactis*.

[b] EF, translation elongation factor 1α gene

[c] CAM, calmodulin gene

[d] *RPB2*, second largest subunit of RNA polymerase II gene

[e] TUB, β-tubulin gene

[f] MAT, mating type *MAT1-1* or *MAT1-2*

[g] ND: not determined

Table 3. Growth rates and morphological characteristics of representative isolates of *Fusarium lactis* species complex multilocus sequence types (ST1 to ST9) from sweet pepper and reference isolates of *F. lactis sensu stricto* (ST12).

ST	Representative isolates[a]	growth rate (mm/7 days)		microconidia length (µm)		macroconidia length (µm)		number of septa in macroconidia	
		isolate mean[b]	ST mean[b]	isolate mean	ST mean[b]	isolate mean[b]	ST mean[b]	isolate mean[b]	ST mean[b]
ST1	MUCL 51511	24.0 ± 1.4 efgh	**22.3** ± 2.5 bc	6.7 ± 1.4 ab	**6.6** ± 0.1 a	46.3 ± 4.4 cd	**45.6** ± 1.0 ab	4.0 ± 0.5 c	**3.9** ± 0.3 abc
	MUCL 51524	20.5 ± 0.7 ijk		6.5 ± 1.1 ab		44.8 ± 4.4 d		3.7 ± 0.6 d	
ST2	MUCL 51519	30.0 ± 0.0 bc	**30.5** ± 0.7 a	6.3 ± 0.9 ab	**6.2** ± 0.0 a	48.1 ± 6.3 bc	**46.1** ± 2.9 ab	4.0 ± 0.6 c	**4.2** ± 0.3 ab
	MUCL 51529	31.0 ± 0.0 ab		6.2 ± 0.8 ab		44.1 ± 4.2 d		4.4 ± 0.7 ab	
ST3	MUCL 51527	27.0 ± 0.0 cde	**26.5** ± 0.7 ab	6.0 ± 0.9 ab	**6.1** ± 0.1 a	35.3 ± 4.2 f	**34.8** ± 0.7 b	3.2 ± 0.5 e	**3.2** ± 0.0 cd
	MUCL 51517	26.0 ± 0.0 def		6.2 ± 0.8 ab		34.3 ± 4.5 ef		3.2 ± 0.5 e	
ST4	MUCL 51523	25.0 ± 0.0 efg	**24.5** ± 0.7 abc	6.3 ± 1.0 ab	**6.4** ± 0.2 a	53.1 ± 5.2 a	**53.6** ± 0.7 a	4.6 ± 0.6 a	**4.6** ± 0.1 a
	MUCL 51818	24.0 ± 1.4 efgh		6.5 ± 1.2 ab		54.1 ± 7.4 a		4.6 ± 0.9 ab	
ST5	MUCL 52692	21.5 ± 0.7 hij	**19.5** ± 2.8 bc	6.7 ± 1.4 ab	**6.3** ± 0.6 a	38.7 ± 5.0 e	**33.0** ± 8.1 b	3.2 ± 0.4 ef	**3.0** ± 0.3 d
	MUCL 52693	17.5 ± 0.7 k		5.9 ± 1.0 ab		27.3 ± 6.1 g		2.8 ± 0.6 f	
ST6	MUCL 52697	20.0 ± 0.0 k	**21.0** ± 2.8 bc	6.0 ± 0.9 ab	**6.0** ± 0.0 a	35.6 ± 4.5 ef	–	3.2 ± 0.4 ef	–
	MUCL 52804	22.0 ± 0.0 ghij		6.0 ± 1.0 ab		NM		NM	
ST7	MUCL 52695	19.5 ± 0.7 jk	**18.5** ± 1.4 c	6.1 ± 1.0 ab	**6.2** ± 0.0 a	NM	–	NM	–
	MUCL 52801	17.5 ± 0.7 k		6.2 ± 1.0 ab		NM		NM	
ST8	MUCL 52696	29.0 ± 1.4 bcd	–	6.7 ± 1.4 a	–	49.0 ± 7.6 b	–	4.3 ± 0.9 b	–
ST9	MUCL 52803	34.0 ± 0.0 a	–	5.8 ± 0.9 b	–	NM	–	NM	–
ST12	[T] MUCL 51854	20.5 ± 0.7 ijk	**22.0** ± 2.1 bc	6.7 ± 1.2 ab	**6.5** ± 0.3 a	45.9 ± 5.9 cd	–	3.1 ± 0.4 ef	–
	MUCL 51855	23.5 ± 0.7 fghi		6.2 ± 1.0 ab		NM		NM	

[a] MUCL, Mycothèque de l'Université catholique de Louvain, Université catholique de Louvain, Louvain-la-Neuve, Belgium; [T], neotype of *F. lactis*.

[b] Within each column, mean values marked with the same letter are not significantly different (Tukey at ST level tests, P=0.05; Bonferroni at isolate level tests); –: mean not calculated.

[c] NM: no macroconidia formed

Table 4. Average mycotoxin production by representative isolates of *Fusarium proliferatum, F. lactis* species complex multilocus sequence types (ST1 to ST9) and reference isolates of *F. lactis sensu stricto* (ST12) from sweet pepper as analysed by LC-MS/MS.

Fungus	ST	isolate[a]	BEA (µg/kg)		FB_1 (µg/kg)		FB_2 (µg/kg)		FB_3 (µg/kg)	
			pepper	maize	pepper	maize	pepper	maize	pepper	maize
FLASC	ST1	MUCL 51511	145	1,080,000	< LOQ	150	–	–	–	–
	ST1	MUCL 51518	1,030	4,280,000	–	–	–	–	–	–
	ST1	MUCL 51524	576	1,130,000	–	219	–	–	–	–
	ST2	MUCL 51516	94	1,550,000	–	1,800	–	89	–	122
	ST2	MUCL 51519	739	783,000	< LOQ	221	< LOQ	–	< LOQ	–
	ST2	MUCL 51529	773	1,080,000	–	1,560	–	127	–	129
	ST3	MUCL 51527	–	723	–	150	–	–	–	–
	ST3	MUCL 51517	–	619	–	211	–	–	–	–
	ST3	MUCL 51522	–	3,220	–	216	–	–	–	–
	ST4	MUCL 51523	645	289,000	–	167	–	–	–	–
	ST4	MUCL 51818	584	380,000	–	287	–	–	–	–
	ST5	MUCL 52692	–	204	–	–	–	–	–	–
	ST5	MUCL 52693	11,400	57,300	–	–	–	–	–	–
	ST5	MUCL 52694	–	484	–	–	–	–	–	–
	ST6	MUCL 52697	161,000	97,300	–	–	–	–	–	–
	ST7	MUCL 52695	–	–	–	–	–	–	–	–
	ST8	MUCL 52696	–	65	–	–	–	–	–	–
	ST9	MUCL 52803	–	174	–	–	–	–	–	–
	ST12	[T] MUCL 51854	–	4,630	–	–	–	–	–	–
	ST12	MUCL 51855	–	434	–	–	–	–	–	–
F. proliferatum	-	MUCL 53013	16	223,000	2540	77,000	900	4,710	385	6,370
none (neg. control)	-	-	–	–	–	–	–	–	–	–

[a] MUCL, Mycothèque de l'Université catholique de Louvain, Université catholique de Louvain, Louvain-la-Neuve, Belgium; [T], neotype of *F. lactis*.

BEA, Beauvericin; FB_1, fumonisin B_1; FB_2, fumonisin B_2; FB_3, fumonisin B_3

–: < limit of detection (LOD). In pepper, LOD for BEA = 2.2 µg/kg, for FB_1 = 24 µg/kg, for FB_2 = 16 µg/kg and for FB_3 = 18 µg/kg. In maize the LOD for BEA = 18 µg/kg, for FB_1 = 30 µg/kg, for FB_2 = 41 µg/kg and for FB_3 = 37 µg/kg.

< LOQ: < limit of quantification (LOQ=2*LOD)

Figure legends

Figure 1: One of 12 MPTs inferred from the combined β-tubulin, calmodulin, translation elongation factor 1α (EF-1α) and *RPB2* gene partial sequences from representative isolates of the *Gibberella fujikuroi* species complex, including *F. lactis* species complex (FLASC) isolates from sweet pepper. The trees were rooted by the outgroup method. Bootstrap intervals higher than 75% are indicated. Biogeographic clades defined by O'Donnell et al. (1998) are indicated. The FLASC clade is strictly conserved in the 12 MPTs, as revealed by the strict-rule consensus analysis (data not shown).

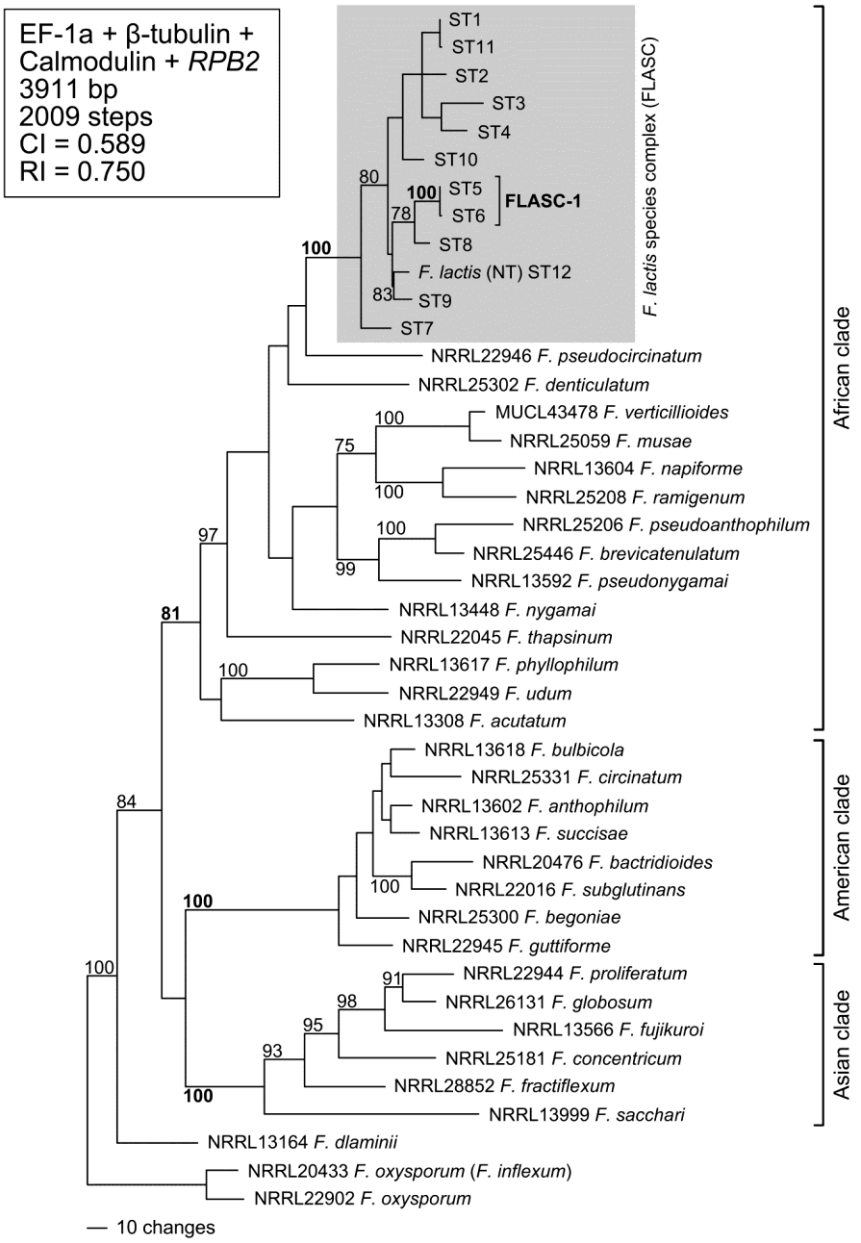

Figure 2: One of three, four, six and two MPTs inferred from the individual translation elongation factor 1α (EF-1 α), calmodulin, *RPB2* and β-tubulin gene partial sequences, respectively, from representative *F. lactis* species complex (FLASC) isolates from sweet pepper. The trees were rooted by the outgroup method. Bootstrap intervals higher than 75% are indicated, except for the 60% bootstrap support of the FLASC clade in the *RPB2* analysis.