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Diversity of *Fusarium* species and mycotoxins contaminating pineapple

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Abstract Pineapple (*Ananas comosus* var. *comosus*) is an important perennial crop in tropical and subtropical areas. It may be infected by various *Fusarium* species, contaminating the plant material with mycotoxins. The aim of this study was to evaluate *Fusarium* species variability among the genotypes isolated from pineapple fruits displaying fungal infection symptoms and to evaluate their mycotoxigenic abilities. Forty-four isolates of ten *Fusarium* species were obtained from pineapple fruit samples: *F. ananatum*, *F. concentricum*, *F. fujikuroi*, *F. guttiforme*, *F. incarnatum*, *F. oxysporum*, *F. polyphialidicum*, *F. proliferatum*, *F. temperatum* and *F. verticillioides*. Fumonisins B_1–B_3, beauvericin (BEA) and moniliformin (MON) contents were quantified by high-performance liquid chromatography (HPLC) in pineapple fruit tissue. Fumonisins are likely the most dangerous metabolites present in fruit samples (the maximum FB_1 content was 250 μg g^{-1} in pineapple skin and 20 μg ml^{-1} in juice fraction). In both fractions, BEA and MON were of minor significance. *FUM1* and *FUM8* genes were identified in *F. fujikuroi*, *F. proliferatum*, *F. temperatum* and *F. verticillioides*. Cyclic peptide synthase gene (*esyn1* homologue) from the BEA biosynthetic pathway was identified in 40 isolates of eight species. Based on the gene-specific polymerase chain reaction (PCR) assays, none of the isolates tested were found to be able to produce trichothecenes or zearalenone.

Keywords *Ananas comosus* · *FUM* genes · Mycotoxins · Phylogeny · Tropical fruit diseases

Introduction

Pineapple [*Ananas comosus* (L.) Merr. var. *comosus*, syn. *Ananas ananas* (L.) Voss] is a crop native to tropical and subtropical areas of South America, where it holds a considerable economic importance (Ploetz 2001). This perennial plant is susceptible to a number of fungal diseases, of which fusariosis is the most severe. The disease affects almost all parts of the plant, damaging particularly fruit and stem apices. However, the agent causing the disease is ambiguous and other diseases caused by *Fusarium* fungi (like fruitlet core rot) have also been reported (Ploetz 2006). As a result of the systemic dispersal inside the host plant, many members of the *Fusarium* genus have the ability of colonising perennial crops with only scarce symptoms of infection while still being detectable (de Oliveira Rocha et al. 2011; Weber et al. 2006). Moreover, even in the case of an asymptomatic infection, significant mycotoxin contamination of the plant tissues is possible (Desjardins 2006; Stankovic et al. 2007; Thiel et al. 1992; von Bargen et al. 2009). *Fusarium guttiforme* has been frequently associated with pineapple fusariosis in Brazil (Ploetz 2006), while *F. subglutinans* emerged as the main cause associated with fruitlet core rot in Hawaii (Rohrbach and Pfeiffer 1976). Both species belong to the *Gibberella fujikuroi* species complex (GFSC); however, *F. subglutinans* is currently not regarded as a pineapple pathogen (Leslie and Summerell

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2006). More recently, a novel species infecting pineapple and originating from South Africa was characterized as *F. ananatum*. The new pathogenic species was found to be closely related to both *F. subglutinans* and *F. guttiforme* (Jacobs et al. 2010). The geographical incidence of those species, as well as their host species range, is still to be determined. It has been demonstrated that, for some pathogens (like *F. oxysporum* and *F. subglutinans*), a considerable level of host–pathogen specificity was developed (Lievens et al. 2007; Steenkamp et al. 1999; Sutherland and Pegg 1992). Recently, Boutigny et al. (2011) found some evidence that members of the *Gibberella zeae* species complex also exhibit host preference to some extent. The analysis of the *tef*-1α sequence is widely used to infer phylogenetic relationships between closely related genotypes (e.g. Jurado et al. 2010; Kristensen et al. 2005; Moretti et al. 2008; Punja et al. 2008; Stępień et al. 2011a, 2011b, 2012). In addition to the *tef*-1α sequence, the sequencing of other polymorphic regions like β-tubulin, *MAT* alleles, H3 histone, cellobiohydrolase-C (*cbh-C*) and topoisomerase II (*topII*) has become increasingly useful for the molecular identification of *Fusaria* (Hatsch et al. 2004; Jacobs et al. 2010; O'Donnell et al. 2004; Steenkamp et al. 1999).

To date, there is little information on secondary metabolites produced by *Fusaria* in infected pineapple plants. Based on the in vitro and also in planta mycotoxin synthesis comparison of the GFSC, one should consider moniliformin (MON), beauvericin (BEA), fumonisins, fusaproliferin, fusarins and fusaric acid as likely major metabolites (Kvas et al. 2009). The fumonisin biosynthetic pathway has been well recognized and described (Proctor et al. 2006) and *FUM* genes (particularly *FUM1*) have often been used for studies of fumonisin-producing fungi (Khaldi and Wolfe 2011; Proctor et al. 2008; Stępień et al. 2011a; Waalwijk et al. 2004). Recently, the structure of the BEA biosynthetic gene cluster in *F. proliferatum* has been revealed (Zhang et al. 2012). Moreover, two clusters responsible for the synthesis of fusarins and fusaric acid by *F. verticillioides* have been elucidated (Brown et al. 2012). The progress in the studies focused on the *Fusarium* secondary metabolite biosynthetic pathways has been recently summarized (Stępień 2013).

Fusarium proliferatum is able to synthesize many of the mycotoxins produced by the members of the *Fusarium* genus. Although, until now, the species was not regarded as a pineapple pathogen (Jacobs et al. 2010; Ploetz 2006), nevertheless, the worldwide distribution of this polyphagous and cosmopolitan species (Kenényi et al. 2002; Jurado et al. 2010) may implicate that its occurrence on pineapple is possible and some isolates have already been described (Stępień et al. 2011a; 2011b). In consequence, a broad range of *Fusarium* mycotoxins would likely occur in pineapple tissues. This group includes fumonisins, which, together with trichothecenes and zearalenone, are regarded as the most dangerous to animal and human health (Desjardins 2006).

The two main aims of this study were to evaluate the species variability of *Fusarium* isolates obtained from pineapple fruit with fungal infection symptoms and to examine their mycotoxigenic potential. The latter was done by (i) quantitative analyses of fumonisins, BEA and MON present in pineapple tissue samples and (ii) identifying essential genes of fumonisin, trichothecene, zearalenone and BEA/enniatin metabolic pathways using gene-specific polymerase chain reaction (PCR) assays.

Materials and methods

Fusarium strains purification

Plant material consisted of commercially available pineapple fruit originating from Costa Rica, Ecuador, Honduras and Hawaii. Additionally, some fruit samples from Indonesia (IN) and Vietnam (VN) were collected directly from the local market in a pineapple breeding area. The pineapple fruits were examined for the presence of *Fusarium* fungi over the course of a 3-year survey. Pieces of fruit tissue were cut out of the pineapple fruit core and plated on potato dextrose agar (PDA) medium. The core is usually the part where mycelia of the contaminating fungi occur first. After a few days of incubation at room temperature, the tips of single hyphae of all *Fusarium* fungi were transferred onto new plates. The obtained fungal strains were passaged onto fresh PDA plates the same way at least three times to assure their purity. For most of the isolates, single spore cultures were performed according to Leslie and Summerell (2006). All isolates are deposited in the KF *Fusarium* collection at the Institute of Plant Genetics, Polish Academy of Sciences, Poznań, Poland.

Colony growth rate measurement

The growth speed of 31 *Fusarium* isolates was measured as the diameter of fungal colonies on 90-mm Petri plates with PDA medium (Oxoid, Basingstoke, Hampshire, UK) following 7 days of incubation in 24-h intervals, replicated twice. The temperature of 25 °C was chosen, as it appeared to be the optimum for differentiation between fast- and slow-growing *Fusarium* strains in previous studies (Stępień et al. 2011b). The mean values were calculated and presented.

DNA extraction, primers, PCR assays and DNA sequencing

Mycelia of the isolates studied were grown on solid PDA medium to control and eliminate any bacterial or fungal

contaminations. They were harvested after 7 days and stored at −20 °C. Genomic DNAs of all isolates were extracted using a hexadecyltrimethylammonium bromide (CTAB) method, as described previously (Stępień et al. 2004), and the DNA concentrations were adjusted to 10 ng μl^{-1}. All the primer details are presented in Table 1. Some of the primers used for PCR assays were designed with Primer3 and PrimerBlast software using sequences of biosynthetic genes deposited in the NCBI GenBank, while others, like *tef*-1α, were previously published and validated. The 25-μl reaction volume consisted of 1 unit of Platinum HotStart Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 1× PCR buffer, 12.5 pmol of each forward and reverse primers, 2.5 mmol l^{-1} of each dNTPs and 10–20 ng of genomic DNA as template. The PCR conditions were as follows: 15 min at 95 °C, 35 cycles of (30–60 s at 94 °C, 30–60 s at 58–63 °C, 1–2 min at 72 °C) and 10 min at 72 °C (see Table 1 for annealing temperatures). Amplicons were electrophoresed in 1.5 % agarose gels (Invitrogen) with ethidium bromide staining and visualised using UV light.

Fragments of *tef*-1α, *FUM1* and *FUM8* genes obtained with the Fum1F1/R2 and Antfum8F/R primers were sequenced. Prior to the sequencing reaction, PCR-amplified DNA fragments were purified using exonuclease I (Epicentre, Madison, WI, USA) and shrimp alkaline phosphatase (Promega, Madison, WI, USA) using the following program: 30 min at 37 °C, followed by 15 min at 80 °C. Both strands were labelled using the BigDye Terminator v3.1 kit (Applied Biosystems, Foster City, CA, USA) and purified by ethanol precipitation according to the procedure outlined by Błaszczyk et al. (2004). Sequence reading was performed using Applied Biosystems 3130 equipment.

Sequence analysis and phylogeny reconstruction

In order to evaluate the phylogenetic relationships between the isolates studied, multiple alignments of *tef*-1α, *FUM1* and *FUM8* sequences were created with ClustalW (Larkin et al. 2007). Subsequently, they were realigned separately for intron and exon regions using MUSCLE (Edgar 2004) and edited in SEAVIEW (Gouy et al. 2010). Phylogenetic relationships were reconstructed using MEGA4 (Tamura et al. 2007). All gene sequences were analyzed using the maximum parsimony approach (closest neighbour interchange heuristics), with complete deletion (i.e. no positions containing gaps were considered in the phylogeny analysis). All reconstructions were validated by bootstrapping with 1,000 replicates.

Aligned sequences of the translation elongation factor *tef*-1α (alignment length of 427 bp) from tested strains were compared to the reference sequences deposited in the NCBI

Table 1 Target genes amplified by polymerase chain reaction (PCR), primer sequences, annealing temperatures (Tm), expected amplicon sizes, GenBank accessions numbers and references

Target gene	Primers	5′>3′ sequence	Tm (°C)/ exp. size	GenBank acc.	References
tef-1α	Ef728M Tef1R	CATCGAGAAGTTCGAGAAGG GCCATCCTTGGAGATACCAGC	63 ~600	Multiple	Stępień et al. (2011a)
FUM1	Fum1F1 Fum1R2	CACATCTGTGGGCGATCC ATATGGCCCCAGCTGCATA	62.5 1,126	AF155773	Stępień et al. (2011a)
FUM8	Antfum8F Antfum8R	ACGGCTCTCCCGTTGTCTGC GGCCAGCCGTCTCTCAAGCG	60 651	AY577456	Stępień et al. (2011a)
PKS4	PKS4_F PKS4_R	AGACGGCGCAACAAGGGCTG GCAGTTGCCCGTGTCGGACA	60 355	AY495638.1	Stępień et al. (2012)
PKS13	PKS13_1 PKS13_2	CCCAGCCAAGCCCAGTACGC ACAGCGGCTGACCTGGGTCA	60 532	DQ019316.1	Stępień et al. (2012)
TRI5	TRI5_1 TRI5_2	AGCGACTACAGGCTTCCCTC AAACCATCCAGTTCTCCATCT	62 545	EF661664.1	Stępień et al. (2011a)
TRI13	TRI13_NIVF TRI13_NIVR	CCAAATCCGAAAACCGCAG TTGAAAGCTCCAATGTCGTG	58 290	AY057841.1	Nicholson et al. (2004)
TRI13	TRI13_DONF TRI13_DONR	CATCATGAGACTTGTKCRAGT GCTAGATCGATTGTTGCATTGAG	58 225	AF336366.2	Nicholson et al. (2004)
esyn1	Esyn_1 Esyn_2	GCCGTTGGCGAGCTGGTCAT GCAAAGCACGCGTCAACGCA	60 997	Z18755.3	Stępień and Waśkiewicz (2013)
bsyn1	beas_1 beas_2	TKGARCAGCGBCAYGAGACM GGWCGRGGGAARTCRGTDGG	58 495	Multiple[a]	Stępień and Waśkiewicz (2013)

[a] Based on consensus sequence elucidated from three NCBI database sequences and three unpublished sequences obtained by the authors in preliminary experiments

GenBank using the BLASTN algorithm for species identification. Fourteen reference strains were also included in the phylogenetic reconstruction: *F. solani* FGSC 9596 (NECHADRAFT_59329), *F. graminearum* NRRL 31084 (FGSG_08811), *F. oxysporum* f. sp. *lycopersici* NRRL 34936 (FOXG_03515), *F. verticillioides* FGSC 7600 (FVEG_02381), *F. ananatum* NRRL 53131 (HM347128), *F. begoniae* NRRL 25300 (AF160293), *F. bulbicola* NRRL 13618 (AF160294), *F. concentricum* NRRL 26434 (AF333933), *F. guttiforme* ITEM 7611 (JN092343), *F. guttiforme* NRRL 22945 (AF160297; re-identified as *F. ananatum* by Jacobs et al. 2010), *F. guttiforme* MRC 6782 (DQ282170), *F. guttiforme* MRC 7539 (DQ282165), *F. subglutinans* MUCL 52468 (HM067691), *F. succisae* NRRL 13613 (AF160291), *F. temperatum* MUCL 52462 (HM067690). The reference sequences for *F. graminearum*, *F. verticillioides* and *F. oxysporum* f. sp. *lycopersici* were taken from the Fungal Genome Initiative (Haas et al. 2011). The reference *F. solani* (Sayers et al. 2012) sequence was obtained from NCBI/RefSeq. All other reference sequences were obtained from NCBI/GenBank. The *F. solani* sequence was used as an outgroup, in accordance with previous reconstructions of *Fusarium* phylogeny demonstrating early divergence of this species (Watanabe et al. 2011).

In the case of the *FUM1* gene, the analysed region was coding (partial 2nd exon of the gene; ca. 920 bp) and corresponds to the linker region between ketoacyl synthase and acyltransferase domains, as well as ca. 73 N-terminal amino acids of the acyl transferase domain (domain boundaries predicted based on NCBI/CDD matches—Marchler-Bauer et al. 2011). For *FUM8*, a shorter region of ca. 620 bp was successfully amplified and sequenced. The region covered a stretch of sequence including both coding and non-coding regions (full 4th and partial 5th exons, as well as partial 3rd and full 4th intron sequences). Additional reference sequences were included, in order to verify the monophyly of *F. proliferatum* biosynthetic genes. These represented known fumonisin-producing strains: *F. oxysporum* FRC O-1890 (Proctor et al. 2008) and *F. verticillioides* FGSC 8961 (Proctor et al. 2006).

Preparation of plant tissue samples

Two fruit fractions, pineapple skin and pineapple juice, were prepared for each sample in order to determine the *in planta* mycotoxin content. The skin was freeze-dried, homogenized and subjected to the same extraction procedure as used for rice cultures (see below). In juice preparation, the ripe fruit flesh was frozen at −80 °C, thawed, blended and centrifuged at 11,000 × *g* and 6 °C to recover clear juice, which, after filtering through Whatman no. 4 filter paper, was used for the mycotoxin extraction protocol.

Preparation of rice cultures

Rice cultures (in three repetitions) were prepared for individual *Fusarium* isolates (Kostecki et al. 1999; Moretti et al. 2008). Long-grain rice samples (50 g per flask) were left overnight at room temperature with the addition of 12.5 ml of water and sterilized by autoclaving the next day. Rice samples were subsequently inoculated with 4 cm^2 of 7-day-old mycelium on PDA medium. The average culture humidity was kept at around 30 % and maintained for 14 days at 25 °C. Afterwards, cultures were dried at room temperature for 48 h.

Mycotoxin quantification

Fumonisins B_1, B_2 and B_3

Sample extracts were prepared from the dry 2-week-old rice cultures and pineapple skin fractions. Five grammes of each sample were homogenized for 3 min in 10 ml of methanol–water (3:1, v/v) and filtered through Whatman no. 4 filter paper. The detailed procedure of extraction and purification of FB analogues was reported earlier (Waśkiewicz et al. 2010). Purified fumonisins content of methanol extracts and pineapple juice was measured using the high-performance liquid chromatography (HPLC) method (Waśkiewicz et al. 2010). A Waters 2695 apparatus (Waters Division of Millipore, Milford, MA, USA) with an X-Bridge column (3.9×100 mm) and a Waters 2475 fluorescence detector (λ_{Ex}=335 nm and λ_{Em}=440 nm) were used. The detection limits were 10 ng g^{-1} for FB_1–FB_3. Preliminary, positive results (on the basis of retention time) were confirmed by thorough HPLC analysis and compared with the relevant calibration curves (the correlation coefficients for FB_1, FB_2 and FB_3 were 0.9967, 0.9981 and 0.9966, respectively). Recoveries for FB_1, FB_2 and FB_3 were 93, 97 and 87 %, respectively. Recovery coefficients were measured in triplicate by extracting the mycotoxins from blank samples spiked with 10–100 ng g^{-1} of the compound. The relative standard deviations for all fumonisins were less than 8 %.

Beauvericin and moniliformin

Samples (15 g) of rice cultures and pineapple skin were homogenized with 75 ml of acetonitrile:methanol:water (16:3:1, v/v/v) and filtered (Whatman no. 4 filter paper). Pineapple juice samples were also filtered. The filtrate was defatted twice with 25 ml of heptane. The bottom layer was evaporated to dryness and the resulting residue subsequently dissolved in 50 ml of methanol:water (55:45, v/v) and extracted twice with 25 ml of dichloromethane. The aqueous, bottom phase (containing MON) was concentrated to

1 ml, while the CH_2Cl_2 phase (containing BEA) was evaporated to dryness and purified according to the method described by Kostecki et al. (1999).

BEA and MON were quantified using a Waters 2695 apparatus with a C18 Nova Pak column (3.9×150 mm for BEA and 3.9×300 mm for MON) and a Waters 2996 Photodiode Array Detector (Waters, Milford, MA, USA) (λ_{max}= 205 nm for BEA and λ_{max}=229 nm for MON). The HPLC conditions for BEA included a constant flow rate of 0.6 ml min^{-1} and acetonitrile:water (85:15, v/v) was used as the mobile phase. The detection limit for BEA was 10 ng g^{-1}. Acetonitrile:water (15:85, v/v) buffered with 10 ml 0.1 M K_2HPO_4 in 40 %*t*-butyl-ammonium hydroxide in 1 L of solvent was used as the mobile phase for MON analysis (flow rate 0.6 ml min^{-1}), with the detection limit set at 25 ng g^{-1}. Positive results (on the basis of retention time) were confirmed by HPLC analysis and comparison with the relevant calibration curve (the correlation coefficients for BEA and MON were 0.9970 and 0.9988, respectively). Recoveries for BEA and MON were 91 and 94 %, respectively, which were measured in triplicate by extracting the mycotoxins from blank samples spiked with 10–100 ng g^{-1} of the compounds. The relative standard deviation values were less than 7 % for BEA and less than 5 % for MON.

Results

Strain isolation, molecular identification and phylogeny

Forty-four *Fusarium* strains were isolated and purified during this study. Some plant samples yielded more than one individual, but only single strains of a species were subjected to subsequent analyses. To assure that the strains are pure and unique, a single-spore subculturing was performed where possible using the procedure described by Leslie and Summerell (2006). This was not possible for all strains, as some of them did not develop spores under laboratory conditions (results not shown).

Partial sequences of the translation elongation factor gene (*tef*-1α) from the strains tested were compared to the reference sequences deposited in the NCBI GenBank using BLASTN. Ten *Fusarium* species were identified: *F. ananatum*, *F. concentricum*, *F. fujikuroi*, *F. guttiforme*, *F. incarnatum*, *F. oxysporum*, *F. polyphialidicum*, *F. proliferatum*, *F. temperatum* and *F. verticillioides*. *F. proliferatum* and *F. ananatum* were identified with the highest frequencies (15 and 14 isolates, respectively). Only single occurrences of *F. fujikuroi*, *F. guttiforme*, *F. temperatum* and *F. verticillioides* species were observed (Fig. 1 and Table 2). Most of the isolates originated from Costa Rica and Ecuador, with only a few samples being from Hawaii, Honduras, Indonesia and Vietnam. Phylogenetic relationships between the isolates were reconstructed (Fig. 1), including the reference sequences of model strains of *F. graminearum*, *F. oxysporum*, *F. verticillioides* and *F. solani*, as well as several other accessions of closely related species (see the Materials and Methods section).

The phylogeny reconstruction for the *tef*-1α fragment supports clear, early divergence of *F. polyphialidicum* strains from the bulk of the considered species. The reconstructed phylogeny shows moderate support for two clades for the majority of all the analyzed *F. proliferatum* strains, except for KF 3408 (two clades with supports of 66 and 56 %, respectively).

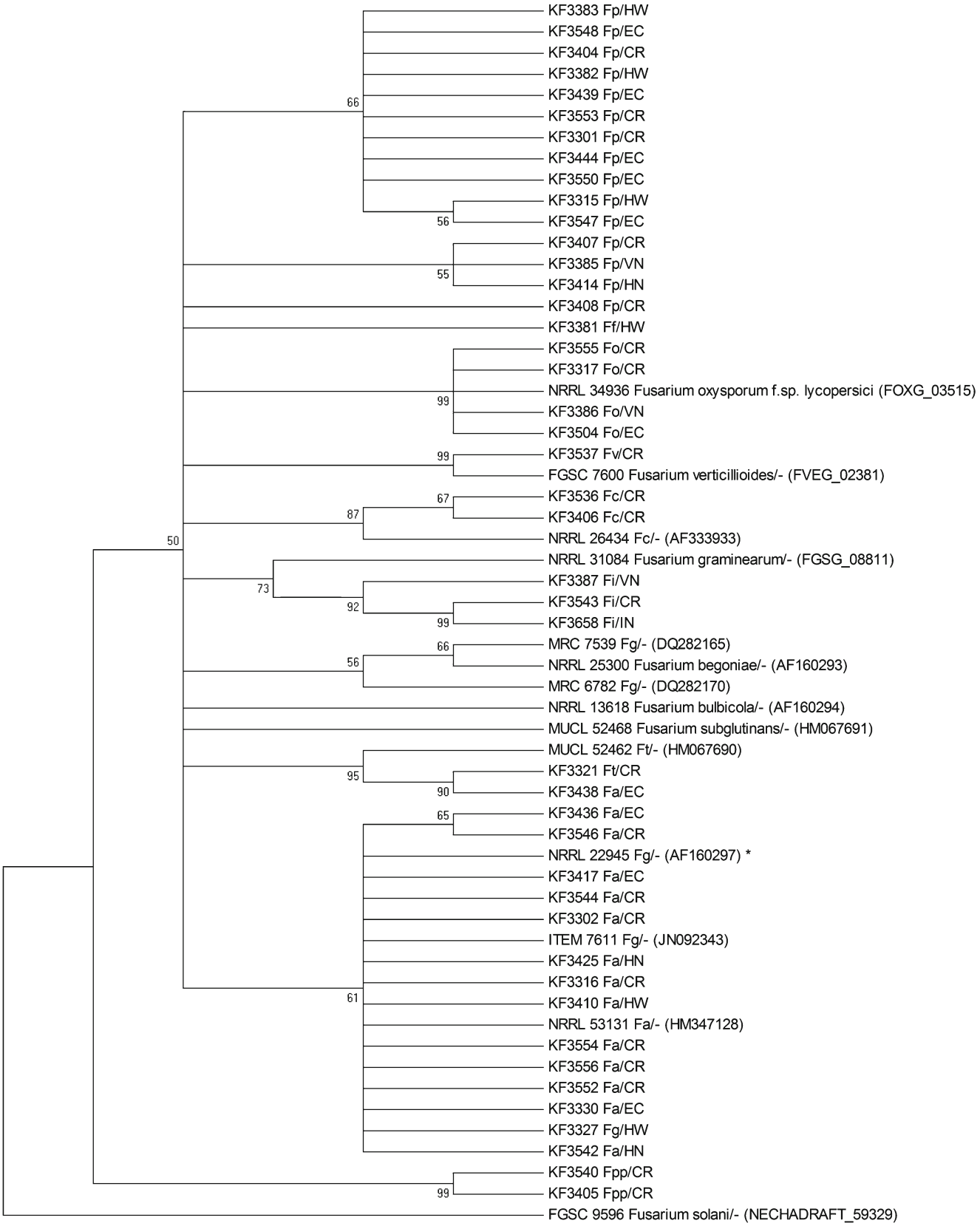
Notably, *F. guttiforme* descent is unresolved in this phylogeny. Some *F. guttiforme* strains (KF 3327, NRRL 22945, ITEM 7611) appear to share common descent with *F. ananatum* (61 % bootstrap support), while grouping with *F. begoniae* NRRL 25300 is implied for two other strains (MRC 6782 and MRC 7539). This suggests the possible misidentification of KF 3327 and ITEM 6711 strains in their respective collections, as some of the model *F. guttiforme* strains (e.g. NRRL 22945) have been previously re-identified as *F. ananatum* in the paper describing this recently characterized species (Jacobs et al. 2010).

Growth speed

Thirty-one *Fusarium* isolates representing ten identified species were subjected to the analysis of growth speed at 25 °C. *F. incarnatum* and *F. concentricum* strains formed the colonies with the greatest speed and *F. guttiforme* and *F. polyphialidicum* were the species for which colony size increase was observed with the lowest rates. To maximize the clarity of the results obtained, only one isolate per species was included (Fig. 2), except for two isolates of *F. ananatum*, *F. polyphialidicum* and *F. proliferatum*, samples which delineate the limits of intra-specific differences observed for growth speeds.

Identification of mycotoxin biosynthetic genes

The presence of *FUM1* and *FUM8* genes (encoding the polyketide synthase and PLP-dependent aminotransferase from the fumonisin biosynthetic gene cluster, respectively) was confirmed in the *F. fujikuroi*, *F. proliferatum*, *F. temperatum* and *F. verticillioides* strains, though with minor exceptions, namely, the *F. fujikuroi* KF 3381 strain gave no amplification for *FUM1* and a single strain of *F. ananatum* (KF 3316) amplified the *FUM1* gene marker with low efficiency. The markers for trichothecene (*TRI5* encoding the trichodiene synthase and *TRI13* encoding the P450 monooxygenase determining deoxynivalenol and nivalenol chemotypes), as well as two zearalenone biosynthetic genes


KF3383 Fp/HW
KF3548 Fp/EC
KF3404 Fp/CR
KF3382 Fp/HW
KF3439 Fp/EC
KF3553 Fp/CR
KF3301 Fp/CR
KF3444 Fp/EC
KF3550 Fp/EC
KF3315 Fp/HW
KF3547 Fp/EC
KF3407 Fp/CR
KF3385 Fp/VN
KF3414 Fp/HN
KF3408 Fp/CR
KF3381 Ff/HW
KF3555 Fo/CR
KF3317 Fo/CR
NRRL 34936 Fusarium oxysporum f.sp. lycopersici (FOXG_03515)
KF3386 Fo/VN
KF3504 Fo/EC
KF3537 Fv/CR
FGSC 7600 Fusarium verticillioides/- (FVEG_02381)
KF3536 Fc/CR
KF3406 Fc/CR
NRRL 26434 Fc/- (AF333933)
NRRL 31084 Fusarium graminearum/- (FGSG_08811)
KF3387 Fi/VN
KF3543 Fi/CR
KF3658 Fi/IN
MRC 7539 Fg/- (DQ282165)
NRRL 25300 Fusarium begoniae/- (AF160293)
MRC 6782 Fg/- (DQ282170)
NRRL 13618 Fusarium bulbicola/- (AF160294)
MUCL 52468 Fusarium subglutinans/- (HM067691)
MUCL 52462 Ft/- (HM067690)
KF3321 Ft/CR
KF3438 Fa/EC
KF3436 Fa/EC
KF3546 Fa/CR
NRRL 22945 Fg/- (AF160297) *
KF3417 Fa/EC
KF3544 Fa/CR
KF3302 Fa/CR
ITEM 7611 Fg/- (JN092343)
KF3425 Fa/HN
KF3316 Fa/CR
KF3410 Fa/HW
NRRL 53131 Fa/- (HM347128)
KF3554 Fa/CR
KF3556 Fa/CR
KF3552 Fa/CR
KF3330 Fa/EC
KF3327 Fg/HW
KF3542 Fa/HN
KF3540 Fpp/CR
KF3405 Fpp/CR
FGSC 9596 Fusarium solani/- (NECHADRAFT_59329)
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◀ **Fig. 1** The most parsimonious tree created on the basis of translation elongation factor 1α (*tef*-1α) sequences of 44 isolates of ten *Fusarium* species, as well as 14 additional sequences from publicly available model strains. *F. solani* (*H. haematococca* MP VI) was used as an outgroup in the analysis. The consistency index for the first most parsimonious tree was 0.587, and the retention index was 0.803. A total of 277 positions were analysed (complete deletion of gapped positions from the initial alignment of 427 base pairs), of which 69 were parsimony informative. The tree was obtained using the maximum parsimony approach (closest neighbour interchange with search level 3 based on ten replicates for the initial tree construction) and tested by bootstrapping (1,000 replicates) with a cut-off value of 50 %. Abbreviations used for the species/country of origin: *Fa F. ananatum*; , *Ff F. fujikuroi*; *Fg F. guttiforme*; *Fi F. incarnatum*; *Fo F. oxysporum*; *Fpp F. polyphialidicum*; *Fp F. proliferatum*; *Ft F. temperatum*; *Fv F. verticillioides*; *CR* Costa Rica; *EC* Ecuador; *HA* Hawaii; *HO* Honduras; *IN* Indonesia; *VN* Vietnam. For 12 model strains, either gene identifiers (from BROAD/JGI model sequences of *F. graminearum*, *F. verticillioides*, *F. oxysporum*, *F. solani*) or GenBank accession codes (all other cases) are given in parentheses. **F. guttiforme* NRRL 22945 subsequently re-identified as *F. ananatum* by Jacobs et al. (2010); see also the discussion of the *tef*-1α phylogeny in the Results section

(*PKS4* and *PKS13* encoding two polyketide synthases), were not found in any of the analysed samples (data not shown). A weak amplification of *TRI13* fragment specific for nivalenol producers was observed in the case of DNA extracted from pineapple skin sample Ecu1_s. Sequencing of the fragment was confirmed to be the P450 monooxygenase from the *TRI* cluster of the *F. graminearum* variant (253 nucleotides with 99 % identities). Although it was not possible to purify any *F. graminearum* isolate from this plant material, still, the skin sample contained trace amounts of nivalenol (19.13 ng g^{-1}), which suggests the presence of the pathogen. Using an *esyn1* gene-specific marker, it was not possible to confirm the presence of the target gene. However, an additional pair of primers was used to amplify the sequence from BEA-producing species (Table 1). All isolates except for three *F. incarnatum* and one *F. verticillioides* had the gene present (Table 2). Partial sequences of *FUM1* and *FUM8* genes from all isolates with the genes present were examined to verify their origin and cross-reference the toxigenic capability with inferred species (assignments based on the *tef*-1α sequence). The only exceptions were isolates KF 3316 (*F. ananatum*) and KF 3414 (*F. proliferatum*), for which neither *FUM1* nor *FUM8* sequences could not be obtained, in spite of their successful amplification (Table 2). Phylogenetic trees were constructed, bootstrapped and topology visualized with not less than 50 % of support (Figs. 3 and 4).

Notably, the divergence of *F. proliferatum* and *F. verticillioides*/*F. oxysporum* (Waalwijk et al. 2004) is well supported by both *FUM1* and *FUM8* phylogenies. The respective reconstructions also demonstrate close relationships between singular *F. temperatum* (*FUM1* and *FUM8*) and *F. fujikuroi* (*FUM8*) genes with the *F. proliferatum* genes (>90 % bootstrap support values for clades).

Mycotoxins synthesized in planta and in vitro

The mycotoxins of three groups, BEA, MON and fumonisins B_1, B_2 and B_3, were quantified in five samples of pineapple juice and skin fractions originating from Ecuador and Costa Rica. To investigate the mycotoxigenic abilities of individual isolates, the cultures of sterilized rice were used. Only a small group of isolates could not be tested; being highly susceptible to the storage conditions (nine isolates out of 44 studied), the mycelia were not viable after a few months of cold storage at −20 °C.

The inter-specific variability of mycotoxigenic abilities displayed by the set of isolates studied is presented in Table 3. To facilitate the comparison of *in planta* versus in vitro mycotoxin contents, isolates purified from analyzed pineapple fruit samples were separated from the others and appended in Table 4.

Discussion

Recent studies reported mainly *F. guttiforme* as the causative agent of pineapple fusariosis (Ploetz 2006). Nevertheless, Jacobs et al. (2010) demonstrated *F. ananatum* as the emerging species involved in pineapple fruit lesions in South Africa. In the present study, numerous *Fusarium* species were obtained from pineapple plant samples besides the two above-mentioned species, namely, *F. concentricum*, *F. fujikuroi*, *F. incarnatum*, *F. oxysporum*, *F. polyphialidicum*, *F. proliferatum*, *F. temperatum* and *F. verticillioides*. In several cases, two different species were identified in the same plant sample (Table 4). The majority of the species occurred with low frequencies (see Table 2) and have not been reported as pineapple pathogens, thus, inoculation experiments are needed in order to confirm the ability of those strains to infect and colonize the pineapple tissue. Taking into account that the harvested pineapple fruit is usually protected from pathogen infiltration and also from colonization by saprophytic agents by spraying a fungicide (like Bayleton), this finding could be helpful in corroborating the supposition that the identified *Fusaria* are not necessarily exogenous contaminants introduced during transport and storage.

The high incidence of *F. proliferatum* deserves significant attention. Recently, Stępień et al. (2011b) characterized the genetic diversity of several strains obtained from pineapple in respect to genotypes obtained from different hosts, showing significant intra-specific diversity in relation to the host of origin. Pineapple-derived strains grouped firmly together, apart from garlic, maize, rice and asparagus strains (Stępień et al. 2011b). The co-occurrence of multiple

Table 2 Forty-four isolates of ten *Fusarium* species purified from contaminated pineapples, their origin, year of isolation and the presence of *FUM1* and *FUM8* genes from the fumonisin biosynthetic pathway and *bsyn1* from the beauvericin (BEA) biosynthetic pathway

Isolate	Species	Year	Origin	*FUM1*	*FUM8*	*bsyn1*
KF 3302[a]	*F. ananatum*	2008	Costa Rica	−	−	+
KF 3316[a]	*F. ananatum*	2008	Costa Rica	+ (weak)	−	+
KF 3330	*F. ananatum*	2008	Ecuador	−	−	+
KF 3410	*F. ananatum*	2009	Hawaii	−	−	+
KF 3417	*F. ananatum*	2010	Ecuador	−	−	+
KF 3425	*F. ananatum*	2009	Honduras	−	−	+
KF 3436	*F. ananatum*	2010	Ecuador	−	−	+
KF 3438	*F. ananatum*	2010	Ecuador	−	−	+
KF 3542	*F. ananatum*	2010	Honduras	−	−	+
KF 3544	*F. ananatum*	2009	Costa Rica	−	−	+
KF 3546	*F. ananatum*	2011	Costa Rica	−	−	+
KF 3552	*F. ananatum*	2011	Costa Rica	−	−	+
KF 3554	*F. ananatum*	2011	Costa Rica	−	−	+
KF 3556	*F. ananatum*	2011	Costa Rica	−	−	+
KF 3406	*F. concentricum*	2009	Costa Rica	−	−	+
KF 3536	*F. concentricum*	2010	Costa Rica	−	−	+
KF 3381	*F. fujikuroi*	2009	Hawaii	−	+	+
KF 3327	*F. guttiforme*	2008	Hawaii	−	−	+
KF 3387[a]	*F. incarnatum*	2009	Vietnam	−	−	−
KF 3543[a]	*F. incarnatum*	2009	Costa Rica	−	−	−
KF 3658[a]	*F. incarnatum*	2011	Indonesia	−	−	−
KF 3317	*F. oxysporum*	2008	Costa Rica	−	−	+
KF 3386	*F. oxysporum*	2009	Vietnam	−	−	+
KF 3504	*F. oxysporum*	2010	Ecuador	−	−	+
KF 3555	*F. oxysporum*	2011	Costa Rica	−	−	+
KF 3405[a]	*F. polyphialidicum*	2009	Costa Rica	−	−	+
KF 3540[a]	*F. polyphialidicum*	2010	Costa Rica	−	−	+
KF 3301	*F. proliferatum*	2008	Costa Rica	+	+	+
KF 3315	*F. proliferatum*	2008	Hawaii	+	+	+
KF 3382	*F. proliferatum*	2009	Hawaii	+	+	+
KF 3383	*F. proliferatum*	2009	Hawaii	+	+	+
KF 3385	*F. proliferatum*	2009	Vietnam	+	+	+
KF 3404	*F. proliferatum*	2009	Costa Rica	+	+	+
KF 3407	*F. proliferatum*	2009	Costa Rica	+	+	+
KF 3408	*F. proliferatum*	2009	Costa Rica	+	+	+
KF 3414	*F. proliferatum*	2010	Honduras	+	+	+
KF 3439	*F. proliferatum*	2010	Ecuador	+	+	+
KF 3444	*F. proliferatum*	2010	Ecuador	+	+	+
KF 3547	*F. proliferatum*	2011	Ecuador	+	+	+
KF 3548	*F. proliferatum*	2011	Ecuador	+	+	+
KF 3550	*F. proliferatum*	2011	Ecuador	+	+	+
KF 3553	*F. proliferatum*	2011	Costa Rica	+	+	+
KF 3321	*F. temperatum*	2008	Costa Rica	+	+	+
KF 3537	*F. verticillioides*	2010	Costa Rica	+	+	−

[a]Strains for which single spore subcultures were not performed

Fusarium genotypes in a single plant sample can often result in a contaminated culture. However, the purification procedure and the acquisition of the *tef*-1α sequence from all the *Fusarium* strains studied serves as a strong, though still indirect, proof of culture purity. The phylogenetic analysis of our isolate set revealed a high similarity level among the

collection of 14 obtained *F. ananatum* isolates, as well as their close relationship with *F. guttiforme*. The latter finding supports well the results presented by Jacobs et al. (2010). Moreover, several other species appeared as being closely related to *F. ananatum* and *F. guttiforme*: *F. begoniae*, *F. bulbicola*, *F. succisae*, *F. subglutinans* and *F. temperatum*, the last of which was described recently by Scauflaire et al. (2011). The population of 16 isolates belonging to *F. proliferatum* appeared as being closely related to *F. fujikuroi* (Fig. 1). No correlation was observed between the country of origin and the species distribution.

After 4 days of isolate cultivation, the colonies measured from 20 to 51 mm and after 7 days from 40 to 80 mm (Fig. 2). The fastest-growing species appeared to be *F. concentricum* and *F. incarnatum*. On the other hand, the slowest-growing strains were isolates of *F. guttiforme* and *F. polyphialidicum*. Considerable intraspecific variance was found in other species, particularly in the cases of *F. ananatum* and *F. proliferatum*, where the differences in the colony diameter between the fastest- and the slowest-growing isolates after 7 days of culturing reached 15 mm (Fig. 2). Thus, the speed of growth could not be used as a species discriminatory factor. What is more likely, the studied species differ in mycotoxigenic potential, and this particular question was chosen as the subject for further research. Several gene-specific PCR assays have been performed to identify the essential genes of the respective metabolic pathways in order to validate the ability of the examined isolates to synthesise other groups of mycotoxins. These included *TRI5* and *TRI13* genes from the trichothecene biosynthetic cluster, *PKS4* and *PKS13* genes from the zearalenone cluster and *esyn1*-encoding enniatin synthase. None of the genotypes tested had the marker fragments present. However, an *F. graminearum TRI13* gene fragment specific for nivalenol producers was amplified using a DNA template extracted from the pineapple skin sample Ecu1_s. Although it was not possible to purify any *F. graminearum* isolate from this plant material, the tested sample contained 19.13 ng g^{-1} of nivalenol. This particular finding suggests that the approach utilizing PCR-based identification of specific biosynthetic genes to predict the presence of mycotoxins is very sensitive, even for such complex matrices as uncultured strains present in the plant material. Concerning cyclic peptide biosynthesis, it was already proven that enniatins and BEA share a common metabolic pathway (Nicholson et al. 2004), and, besides enniatins, the *esyn1* gene is also involved in BEA biosynthesis. Previous studies have already shown the sequence divergence between enniatin versus BEA-producing strains (Stępień and Waśkiewicz 2013). Therefore, an additional primer pair was used to amplify a portion of the *esyn1* homologue from BEA-producing species (Table 1) and the gene was identified in 40 out of 44 isolates studied, suggesting the ability of those strains to synthesize BEA (Table 2). All isolates of *F. proliferatum*, *F. fujikuroi*, *F. verticillioides* and *F. temperatum* had both *FUM1* and *FUM8* genes successfully verified by amplification, except for *FUM1* in *F. fujikuroi* (KF 3381) and a single isolate of *F. proliferatum* (KF 3414), for which the sequence could not be read. The outliers can be explained by the interspecific sequence divergence and are consistent with the

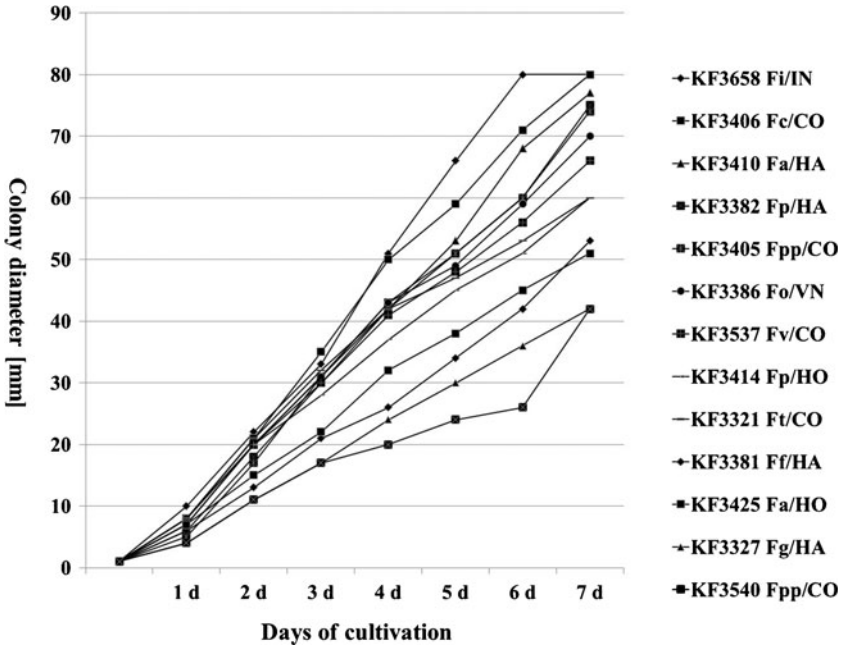

Fig. 2 Colony diameter of the 13 chosen strains of ten *Fusarium* species measured in 24-h intervals on potato dextrose agar (PDA) medium at 25 °C. Two strains of *F. ananatum* (KF 3410 and 3425), *F. polyphialidicum* (KF 3405 and 3540) and *F. proliferatum* (KF 3382 and 3314) were included to show the intraspecific variance. Abbreviations used for the species/country of origin: *Fa F. ananatum*; *Fc F. concentricum*; *Ff F. fujikuroi*; *Fg F. guttiforme*; *Fi F. incarnatum*; *Fo F. oxysporum*; *Fpp F. polyphialidicum*; *Fp F. proliferatum*; *Ft F. temperatum*; *Fv F. verticillioides*; *CO* Costa Rica; *HA* Hawaii; *HO* Honduras; *IN* Indonesia; *VN* Vietnam

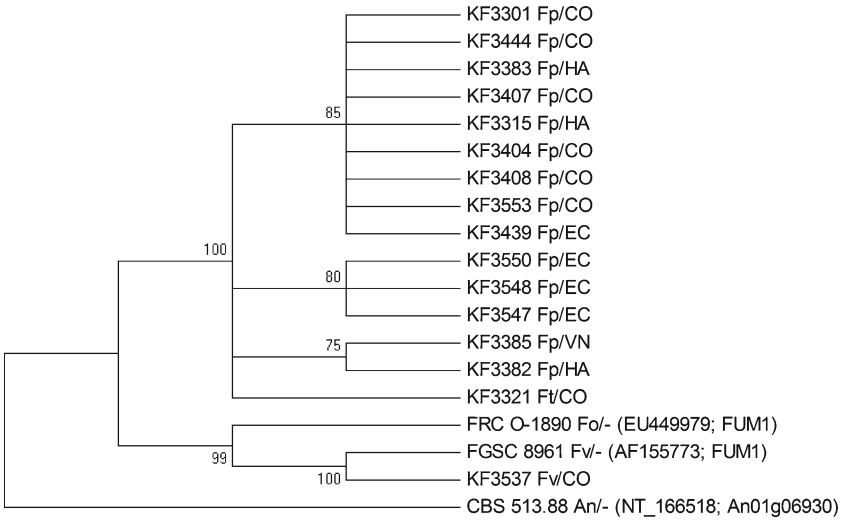

Fig. 3 Consensus phylogenetic tree obtained based on the partial *FUM1* gene sequences of *F. proliferatum*, *F. temperatum* and *F. verticillioides* isolates used in the study (16 isolates). The *FUM1* homologue from *Aspergillus niger* CBS 513.88 was used as an outgroup. Additional sequences from FGSC 8961 (*F. verticillioides*) and FRC O-1890 (*F. oxysporum*) were also included in the analysis. The tree was obtained using the maximum parsimony approach (CNI level 3, with ten replicates for the initial tree construction) and tested by bootstrapping (1,000 replicates) with a cut-off value of 50 %. The consistency index for the first most parsimonious tree was 0.896 and the retention index was 0.923. A total of 555 positions were analysed, of which 116 were parsimony informative. Abbreviations used for the species/country of origin: *An Aspergillus niger*; *Fp F. proliferatum*; *Ft F. temperatum*; *Fv F. verticillioides*; *CO* Costa Rica; *EC* Ecuador; *HA* Hawaii; *HO* Honduras; *IN* Indonesia; *VN* Vietnam. For GenBank reference sequences, both the accession number and locus tag are quoted in parentheses

previous work, where, using a different pair of primers, it was possible to identify the *FUM1* gene (Stępień et al. 2011b). In both the *FUM1* and *FUM8* phylogenies, reference sequences of homologues from *Aspergillus niger* strain CBS 513.88 (Pel et al. 2007) were used as outgroups. The details of the exact evolutionary scenario leading to the emergence of the functional fumonisin cluster in both *A. niger* and *Fusarium* sp. are still unresolved, namely,

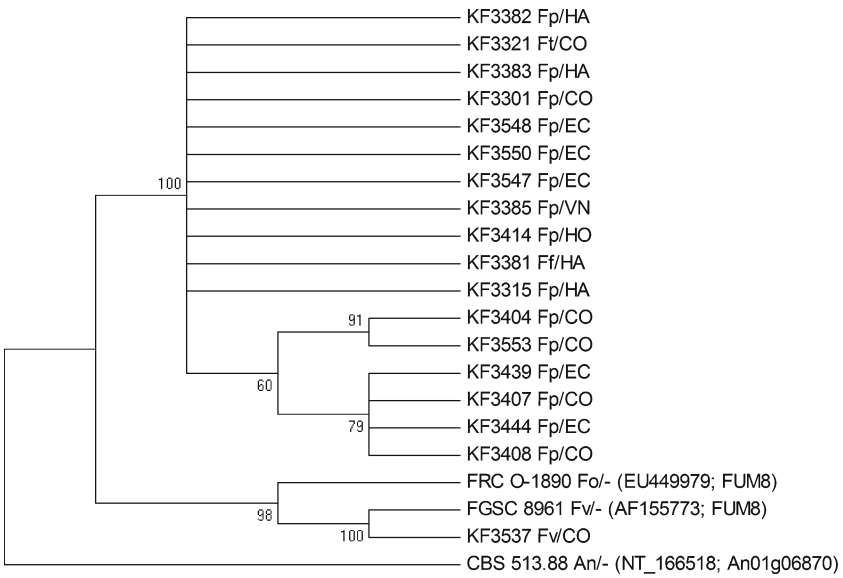

Fig. 4 Consensus phylogenetic tree obtained based on the partial *FUM8* gene sequence of *F. proliferatum*, *F. temperatum* and *F. verticillioides* strains used in the study (18 isolates). The *FUM8* homologue from *Aspergillus niger* CBS 513.88 was used as an outgroup. Additional sequences from FGSC 8961 (*F. verticillioides*) and FRC O-1890 (*F. oxysporum*) were also included in the analysis. The consistency index for the first most parsimonious tree was 0.858 and the retention index was 0.894. The tree was obtained using the maximum parsimony approach (CNI level 3, with ten replicates for the initial tree construction) and tested by bootstrapping (1,000 replicates) with a cut-off value of 50 %. A total of 526 positions was analysed, of which 105 were parsimony informative. Abbreviations used for the species/country of origin: *An Aspergillus niger*; *Ff F. fujikuroi*; *Fp F. proliferatum*; *Ft F. temperatum*; *Fv F. verticillioides*; *CO* Costa Rica; *EC* Ecuador; *HA* Hawaii; *HO* Honduras; *IN* Indonesia; *VN* Vietnam. For GenBank reference sequences, both the accession number and locus tag are quoted in parentheses

Table 3 Means and standard deviations (SDs) of the amounts of fumonisins (FB_1–FB_3), moniliformin (MON) and beauvericin (BEA) concentrations produced in vitro by 28 isolates of nine *Fusarium* species

Isolate	*Fusarium* species	FB_1 (μg/g)	FB_2 (μg/g)	FB_3 (μg/g)	MON (μg/g)	BEA (μg/g)
KF 3302	*F. ananatum*	0.16±0.05	0.09±0.02	0.04±0.01	ND 0	0.42±0.09
KF 3330	*F. ananatum*	9.80±2.21	1.02±0.53	0.32±0.02	0.04±0.01	0.96±0.07
KF 3410	*F. ananatum*	nt	nt	nt	ND	0.70±0.03
KF 3417	*F. ananatum*	nt	nt	nt	ND	4.70±0.56
KF 3425	*F. ananatum*	nt	nt	nt-	ND	5.85±0.87
KF 3438	*F. ananatum*	nt	nt	nt	ND	ND
KF 3436	*F. ananatum*	3.29±0.45	0.95±0.05	0.32±0.02	ND	91.47±11.12
KF 3406	*F. concentricum*	10.41±2.11	ND	0.28±0.04	30.04±4.12	0.51±0.06
KF 3536	*F. concentricum*	9.38±0.97	ND	0.28±0.03	ND	ND
KF 3381	*F. fujikuroi*	1,558.98±187.15	465.96±39.76	81.64±9.76	27.55±3.54	1.65±0.28
KF 3327	*F. guttiforme*	nt	nt	nt	ND	7.70±1.15
KF 3386	*F. oxysporum*	ND	ND	ND	17.23±2.27	ND
KF 3504	*F. oxysporum*	ND	ND	ND	2.22±0.18	3.93±0.55
KF 3405	*F. polyphialidicum*	2.91±0.28	0.41±0.03	0.11±0.02	ND	6.49±1.09
KF 3540	*F. polyphialidicum*	18.87±3.42	4.30±0.37	1.15±0.23	0.02±0.01	6.78±0.75
KF 3301[a]	*F. proliferatum*	1,353.00±154.90	496.00±39.65	133.00±21.98	110.56±9.76	0.83±0.06
KF 3315[a]	*F. proliferatum*	1,820.00±202.11	534.00±21.70	113.00±20.54	59.40±4.44	0.09±0.02
KF 3382	*F. proliferatum*	1,785.76±176.53	450.54±51.33	132.90±18.77	18.40±2.09	3.39±0.28
KF 3383[a]	*F. proliferatum*	930.00±88.69	204.00±017.73	79.00±6.55	158.46±11.63	1.08±0.18
KF 3385[a]	*F. proliferatum*	7.01±0.64	2.16±0.18	0.79±0.04	14.22±1.08	2.26±0.65
KF 3404	*F. proliferatum*	856.39±78.15	330.03±20.18	109.07±9.23	49.31±3.32	3.99±0.57
KF 3407	*F. proliferatum*	2,419.32±199.53	379.86±43.09	139.77±10.44	81.73±7.65	4.15±0.22
KF 3408	*F. proliferatum*	2,686.23±303.41	757.94±54.31	367.82±40.12	27.68±1.72	0.35±0.02
KF 3414	*F. proliferatum*	3,299.01±276.80	855.64±68.98	593.08±49.52	15.98±0.98	41.13±3.74
KF 3439	*F. proliferatum*	1,032.76±97.45	134.60±10.25	96.49±10.01	93.08±10.13	8.61±1.43
KF 3444	*F. proliferatum*	1,568.18±209.83	210.93±15.37	140.35±9.12	65.01±5.43	24.75±3.11
KF 3321	*F. temperatum*	2.25±0.32	0.35±0.04	0.08±0.02	7.45±0.61	12.48±0.89
KF 3537	*F. verticillioides*	59.65±6.16	19.37±0.99	5.86±0.76	0.07±0.01	0.05±0.01

ND not detected; *nt* not tested

[a] Isolates previously described by Stępień et al. (2011a) and/or Stępień et al. (2011b)

the direction of the ancestral horizontal transfer (Khaldi and Wolfe 2011) and dating cluster formation in light of different organization within *F. verticillioides* and *F. proliferatum* (Waalwijk et al. 2004). However, the outgroup choice itself is corroborated by both strong sequence divergence (ca. 55–60 % protein sequence identity between *A. niger* and *Fusarium* genes) and previously mentioned analysis by Khaldi and Wolfe (2011).

To validate the potential risk of pineapple contamination with mycotoxins, BEA, MON and fumonisins contents were measured in fruit tissue samples (Table 4). Also, rice cultures of individual isolates were prepared in order to establish the mycotoxigenic potential of individual genotypes in controlled laboratory conditions (Table 3). Across all the analyzed isolates, *F. proliferatum* appeared to be the species producing the highest amounts of fumonisins (FBs) and MON. Concerning FBs, only one isolate could be qualified as a low-efficiency producer (KF 3385) and only another two yielded less than 1 mg g^{-1} of FB_1 and the most efficient producer (KF 3414) yielded about 3.3 mg g^{-1} of FB_1. The *F. fujikuroi* KF 3381 produced FB_1 in a comparable amount of over 1.5 mg g^{-1} (Table 3). Both *F. temperatum* and *F. verticillioides* are known to produce FBs in significant amounts (Proctor et al. 1999; Scauflaire et al. 2011) and can be described as medium-efficiency producers (between 50 and 100 μg g^{-1} of FB_1 produced) and the remaining species as low-efficiency producers. MON was also produced in the highest amounts by *F. proliferatum* isolates, though, in this case, the yield was not very high, with the highest value of 158 μg g^{-1}. *F. ananatum* isolates did not produce MON, but KF 3436 synthesized BEA in the highest amount (91 μg g^{-1}) of all the isolates tested (Table 3).

Table 4 Means and standard deviations (SD) of the amounts of fumonisins (FB_1–FB_3), moniliformin (MON) and beauvericin (BEA) concentrations measured in five samples of pineapple juice and skin fractions originating from Costa Rica (Cos) and Ecuador (Ecu), together with the mycotoxin yield for seven isolates of three *Fusarium* species purified from the respective samples

Sample ID/Isolate No.	FB_1	FB_2	FB_3	MON	BEA
Pineapple juice samples (μg/ml)					
Cos1_j	8.18±1.07	0.94±0.09	0.17±0.02	0.20±0.01	0.00
Cos2_j	14.85±5.12	1.46±0.32	0.23±0.03	0.04±0.01	0.00
Cos3_j	21.01±4.89	0.74±0.08	0.17±0.03	0.04±0.02	0.00
Cos4_j	23.73±5.22	1.21±0.24	0.18±0.03	0.03±0.01	0.00
Ecu1_j	22.18±3.38	0.95±0.07	0.23±0.04	0.01±0.01	0.00
Pineapple skin samples (μg/g)					
Cos1_s	128.57±11.09	1.27±0.09	0.70±0.05	0.79±0.06	0.31±0.02
Cos2_s	33.23±2.65	0.64±0.05	0.48±0.03	0.02±0.01	0.23±0.02
Cos3_s	33.06±2.88	0.82±0.05	0.74±0.05	0.04±0.02	0.36±0.01
Cos4_s	25.65±1.83	0.59±0.06	0.56±0.04	0.06±0.02	1.60±0.09
Ecu1_s	247.74±20.76	1.48±0.11	0.11±0.02	0.08±0.03	0.79±0.05
Rice cultures of isolates obtained from analyzed material (μg/g)					
KF 3546 (*F. ananatum*/Cos1)	3.62±0.27	0.45±0.05	0.11±0.01	0.06±0.02	2.46±0.34
KF 3552 (*F. ananatum*/Cos2)	10.32±0.87	0.45±0.04	0.39±0.05	0.01±0.01	3.49±0.37
KF 3553 (*F. proliferatum*/Cos3)	2,125.42±154.78	368.79±54.41	189.48±35.09	23.90±6.18	10.54±2.35
KF 3554 (*F. ananatum*/Cos3)	26.05±1.34	5.09±0.72	1.13±0.15	0.03±0.01	1.31±0.11
KF 3555 (*F. oxysporum*/Cos4)	10.97±0.99	6.27±0.55	0.47±0.05	5.22±0.44	3.24±0.45
KF 3556 (*F. ananatum*/Cos4)	3.73±0.41	1.08±0.23	0.47±0.06	0.04±0.01	1.73±0.15
KF 3548 (*F. proliferatum*/Ecu1)	1,861.16±115.76	481.92±34.80	98.40±10.11	97.44±9.98	53.88±7.68

Again, *F. proliferatum* isolates recovered from the analysed plant samples were the most effective FBs, MON and BEA producers. Interestingly, in the original pineapple samples Cos2 and Cos4—samples that did not contain *F. proliferatum* but only *F. ananatum* and *F. oxysporum*—more FBs have been identified *in planta* than in the respective isolates cultured on rice. Such inconsistency might be explained at least two-fold:

(i) An additional unknown FBs producer was present in the infected fruit, but was not identified in cultures, or
(ii) Rice culture used for FBs biosynthesis in vitro was not a suitable medium for those strains causing low biosynthesis level.

In conclusion, concerning a contribution of individual species to the contamination of pineapple with mycotoxins, the role of *F. ananatum*, *F. oxysporum*, *F. guttiforme* and *F. polyphialidicum* as mycotoxin producers can be regarded as limited, while *F. proliferatum* emerges as a major, understated threat. The same conclusion can be drawn from the analysis of *in planta* mycotoxin content results. Pineapple skin from infected fruit contained as much as 250 μg g^{-1} of FB_1 and the metabolite concentration in pineapple juice was about ten times lower. High FB content in the skin samples is likely caused by the concentration of toxins achieved by the application of a freeze-drying step. In both fractions, BEA and MON were of minor significance (Table 4). According to the US Food and Drug Administration (FDA) exposure guidelines, the total fumonisin content in corn-based food products may reach 2–4 ppm. This means that the naturally occurring FB levels in (stored) pineapple fruits are potentially hazardous to human health.

Taken as a whole, the obtained results demonstrate that, of all the species identified, *F. ananatum* and *F. proliferatum* are probably the most commonly occurring *Fusaria* and regarding mycotoxin produced, *F. proliferatum* can be potentially the most dangerous species found in plant tissues of pineapple. Additionally, they serve as a proof of the species' metabolic activity in planta and suggest the need for FBs contamination control in pineapple and, likely, in other tropical crops most often consumed in unprocessed form.

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