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# Molecular identification and mycotoxin production of *Lilium longiflorum*-associated fusaria isolated from two geographic locations in the United States

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**Abstract** *Fusarium* diseases of Liliaceae crops cause significant losses worldwide. Yet some *Fusarium* species are found *in planta* without causing disease or even in a symbiotic relationship with its host. In this study we identified and characterized the *Fusarium* species isolated from soil, and from healthy and diseased bulbs of *Lilium longiflorum* grown in New Jersey and Oregon in the United States. The predominant *Fusarium* species from the Oregon location were *F. solani* (74%) and *F. oxysporum* (20%), whereas *F. concentricum* (43%) and *F. proliferatum* (26%), both belonging to the *Gibberella fujikuroi* species complex (GFSC), were the most commonly isolated species from New Jersey. To our knowledge, this is the first report of *F. concentricum* associated with Liliaceae. All of the isolates were characterized with sequences of the internal transcribed spacer and translation elongation factor 1-alpha genes. The 24 GFSC isolates were further characterized with mating type, mating population, and mycotoxin analysis. Results showed that all GFSC isolates were *MAT-2*, suggesting that the populations may be asexually reproducing in the region examined. The majority of the GFSC isolates produced beauvericin. Enniatin A, B, $B_1$ and fusaproliferin were produced by a few isolates. Enniatin $A_1$ and fumonisins were not detected in any of the isolates. Although *F. oxysporum* and *F. solani* are well-known bulb pathogens, many isolates of *F. oxysporum* and *F. solani*, and all of the *F. concentricum* and *F. proliferatum* were isolated from asymptomatic bulbs, suggesting their endophytic association with lilies.

**Keywords** Easter lily · *Fusarium concentricum* · *Fusarium proliferatum* · Mating type · Phylogeny · TEF

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## Introduction

The cultivation of *Lilium longiflorum*, originally a native of the islands off the coast of Southern Japan, in the United States, is a practice of economic importance. *Lilium longiflorum,* commonly known as the Easter lily, is an important component of the US cut flower ($67 million) and potted lily plant ($27 million) markets (http://usda.mannlib.cornell.edu/usda/current/FlorCrop/FlorCrop-06-04-2010_revision.pdf). The majority of the commercial Easter lily bulb crop is produced in the fields straddling Northern California

(CA) and Southern Oregon (OR) along the Pacific Ocean, although *L. longiflorum* can be grown throughout the temperate zone. The Pacific Bulb Growers Association, based in Brookings, OR produces some of the classic Easter lily varieties such as "Nellie White". The Easter lily breeding program at Rutgers University in New Jersey (NJ) produces new *L. longiflorum* cultivars with higher flower numbers per stem, as well as hybrid cultivars for the garden.

A variety of fungi have been reported to be associated with Easter lilies (Bald and Solberg 1960). Among them, the incidence of *Fusarium*-related infection is of the greatest significance in Easter lily and other bulb crops (Bald et al. 1971). *Fusarium* is a diverse and widely distributed genus with nutritional modes ranging from saprotrophic to pathogenic in nature (Geiser et al. 2004; Proctor et al. 2010; Walsh et al. 2010). Best known for their plant pathogenic and mycotoxin-producing members, there are also endophytic fusaria that live within the plant tissue causing no visible signs of infection. Some of the endophytic fungi can become pathogenic, but only when the host plant is subjected to unfavourable environmental and developmental conditions (Walsh et al. 2010).

*Fusarium oxysporum* is one of the most economically significant members in the genus *Fusarium*. It is a fungus with no known sexual state, heterogeneous and composed of dozens of formae speciales (Leslie and Summerell 2006). It has been implicated in vascular wilt diseases (Beckman 1987) and is the causal agent of the basal bulb and root rot of lilies (Bald et al. 1971). Nevertheless, *F. oxysporum* has also been isolated from asymptomatic roots of food crops and ornamental flower bulbs (Baayen et al. 1998). *Fusarium solani* species complex (FSSC) is another group of fungi associated with a large number of plant species (Burgess and Summerell 1992). It includes more than 45 distinct lineages, ranging from saprophytes to plant and human pathogens (Zhang et al. 2007). The *Gibberella fujikuroi* species complex (anamorph: *Fusarium*) includes at least eight biological species (mating populations) and 32 additional asexual species or phylogenetic lineages that produce a wide range of mycotoxins and secondary metabolites (Steenkamp et al. 2000). Many economically important plants are susceptible to diseases caused by members of this complex, such as the bakanae disease of rice and pitch canker of pines. Given the lack of distinguishing morphological characters among the species, mating tests (Leslie and Summerell 2006) and molecular phylogenetic analysis (O'Donnell et al. 1998) have been successfully used to differentiate the species. The internal transcribed spacer (ITS) of the rRNA genes and the translation elongation factor-1 alpha gene (TEF) are the most frequently used and informative loci for phylogenetic studies of *Fusarium* species (Geiser et al. 2004; O'Donnell et al. 2000).

*Fusarium proliferatum* (teleomorph *G. intermedia*, a member of GFSC) can produce a broad range of toxins, e.g. fumonisins, beauvericin, and moniliformin (Proctor et al. 2010), and is a well-known plant pathogen of agriculturally important crops such as rice (Desjardins et al. 1997), onion and garlic (Stankovic et al. 2007; Palmero et al. 2010). The incidence of *F. proliferatum* in other bulb-producing ornamentals has not been studied in detail so far, but it has been reported to cause rot of *Lilium* crops (Prado-Ligero et al. 2008). There is also evidence to support an endophytic role in wheat (Bishop 2002).

The main objective of this study was to expand knowledge of *Fusarium* species associated with Easter lilies by molecular characterization of the isolates, and to compare species diversity in two distinct geographic locations. Special emphasis was placed on the GFSC isolates, by assessing mating type and mating population, as well as by evaluating their in vitro toxin production capacity, since there have been no studies of these biological species in Easter lilies.

## Materials and methods

### Fungal isolation and identification

*Fusarium* isolates were collected from diseased and healthy bulbs of *L. longiflorum* cultivars 7–4, 4–8 and 7–9, grown in fields and greenhouses in East Brunswick, NJ and from a commercial cultivar (Nellie White) grown in the Smith River region between Brookings OR and Smith River CA. Isolates were also collected from soil samples at both the sampling sites. Sampling was carried out from August 2008 to December 2009 in NJ and in January 2010 in OR. Bulb scales from diseased and healthy bulbs were immersed in 95% ethanol for 1 min followed by a solution of 1% sodium hypochlorite for 1 min.

Samples were then washed three times with sterile water and air-dried in a laminar flow hood. The dried samples were plated on *Fusarium*-selective Peptone PCNB Agar (PPA). Soil fusaria were isolated following the soil dilution plate technique described by Leslie and Summerell (2006). All isolates were single-spored and routinely cultured on low nutrient Spezieller Nahrstoffarmer Agar (SNA). PDA was used to harvest mycelium for DNA extraction. All isolates originating from this study (Table 1) are maintained in the NRRL collection, USDA, Peoria, IL, USA.

## Morphological characterization

Microscopic examination was carried out for strains grown on SNA, with a 2 cm piece of sterile filter paper placed on cooled agar (Nirenberg and O'Donnell 1998). Cultures were incubated for 10–14 days in complete darkness. Petri dishes were viewed under a stereomicroscope to study features of the aerial mycelium. To study the conidial morphology, spores were mounted in distilled water and viewed with a compound light microscope.

## DNA isolation, PCR amplification, and sequencing

DNA was extracted from the mycelia of single spore isolates grown on PDA for 7 days with the Ultraclean Soil DNA Isolation Kit (MO BIO laboratories, CA, USA). ITS and TEF were amplified with the primer sets ITS5/ITS4 (White et al. 1990) and EF1/EF2 (O'Donnell et al. 1998), respectively. PCR was performed in an Applied Biosystems 2620 thermocycler. The amplification reaction mixture (50 μl) for ITS consisted of 20 ng DNA, 0.2 μM of each primers ITS4 and ITS5, 200 μM of dNTPs (Promega, WI, USA), 1.25 U *Taq* DNA polymerase, and 1x GoTAQ green PCR buffer. PCR cycling conditions consisted of an initial denaturation step at 95°C for 45 s, 35 cycles of 95°C for 45 s, 57°C for 45 s, 72°C for 45 s, and a final extension at 72°C for 10 min. The amplification mixture (50 μl) for TEF consisted of 5 ng DNA, 0.2 μM of each primers EF1 and EF2, 200 μM of dNTPs, 1.25 U Amplitaq Gold DNA polymerase (Applied Biosystems, CA, USA) and PCR Gold Buffer with 2.5 mM $Mg^{2+}$. The cycling conditions consisted of an initial denaturation step at 95°C for 6 min, 35 cycles of 95°C for 1 min, 57°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. Diagnostic multiplex PCR for *MAT-1* and *MAT-2* was based on Steenkamp et al. (2000) using primers GFmat1a, GFmat1b, GFmat2c, and GFmat2d. Briefly, a 10 μl reaction contained 10 ng template DNA, 1X PCR buffer (Applied Biosystems, CA, USA), 0.1 μM each of the four primers, 200 μM dNTPs, 2.5 mM $Mg^{2+}$ and 0.4 U of *Taq* polymerase (Applied Biosystems, CA, USA). The cycling conditions consisted of an initial denaturation step at 95°C for 6 min and 35 cycles of 95°C for 1 min, 59°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. PCR detection of the enniatin synthetase gene *esyn1* was conducted using primers esy1 and esy2 (Kulik et al. 2007). PCR conditions were the same as used in ITS-PCR. The PCR products were visualized by gel electrophoresis and the successfully amplified samples were purified with the QIAquick PCR purification kit (QIAGEN, CA, USA) and were then subjected to sequencing, which was carried out by GENEWIZ, Inc. (South Plainfield, NJ, USA).

## Phylogenetic analyses

DNA sequences generated in this study along with the sequences retrieved from the GenBank were aligned using Clustal X 2.0.11 and further adjusted manually upon visual inspection for machine reading errors. Data for each gene was analyzed separately for both the single-locus dataset and for the combined dataset. MrModeltest 2.3 (Nylander 2004) was used to determine the best-fit model of nucleotide substitution. The models chosen by the hierarchical likelihood ratio test (hLRT) and Akaike information criterion (AIC) were used in the subsequent phylogenetic analyses by MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). The Bayesian phylogenetic tree was constructed by running four chains of 1,000,000 generations with the sampling frequency of 1,000 generations. The burn-in was set at 10,000 generations to discard trees obtained before congruence. Bayesian posterior probabilities were calculated based on the 50% majority rule (Barthélemy and McMorris 1986).

Figtree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/) was used to construct the Bayesian tree with posterior probability. Parsimony analysis was performed with tree bisection reconnection (TBR) branch swapping in heuristic searches for the individual and combined datasets using Winclada 1.00.08 and Nona

**Table 1** Isolate number, source, isolation date, ID, geographic origin, and GenBank accession number of the *Fusarium* isolates used in the study

| [a]NRRL | Isolate No | Source | Date isolated | ID | Geographic origin | ITS GenBank accession no | TEF GenBank accession no |
|---|---|---|---|---|---|---|---|
| 54167 | F1 | Healthy bulb | 8/20/2008 | *F. concentricum* | NJ, USA | HQ379633 | JN099734 |
| 54168 | F2 | Diseased bulb | 8/20/2008 | *F. oxysporum* | NJ, USA | HQ379648 | JN167175 |
| 54169 | F3 | Diseased bulb | 8/20/2008 | *F. solani* | NJ, USA | HQ379661 | JN167185 |
| 54170 | F4 | Healthy bulb | 7/28/2008 | *F. concentricum* | NJ, USA | HQ379634 | JN099735 |
| 54171 | F5 | Diseased bulb | 4/1/2009 | *F. oxysporum* | NJ, USA | HQ379656 | JN167178 |
| 54172 | F6 | Diseased bulb | 4/1/2009 | *F. oxysporum* | NJ, USA | HQ379649 | JN167179 |
| 54173 | F7 | Healthy bulb | 12/22/2008 | *F. concentricum* | NJ, USA | HQ379635 | JN099736 |
| 54174 | F8 | Healthy bulb | 3/9/2009 | *F. proliferatum* | NJ, USA | HQ379687 | JN167213 |
| 54175 | F9 | Healthy bulb | 4/3/2009 | *F. concentricum* | NJ, USA | HQ379646 | JN099737 |
| 54176 | F10 | Healthy bulb | 8/20/2008 | *F. concentricum* | NJ, USA | HQ379636 | JN099738 |
| 54177 | F11 | Healthy bulb | 12/22/2008 | *F. concentricum* | NJ, USA | HQ379637 | JN099739 |
| 54178 | F12 | Diseased bulb | 10/31/2008 | *F. solani* | NJ, USA | HQ379662 | JN167188 |
| 54179 | F13 | Healthy bulb | 4/1/2009 | *F. concentricum* | NJ, USA | HQ379638 | JN099740 |
| 54180 | F14 | Healthy bulb | 8/20/2008 | *F. concentricum* | NJ, USA | HQ379639 | JN099741 |
| 54181 | F15 | Healthy bulb | 4/3/2009 | *F. proliferatum* | NJ, USA | HQ379689 | JN167214 |
| 54182 | F16 | Healthy bulb | 4/1/2009 | *F. proliferatum* | NJ, USA | HQ379690 | JN167215 |
| 54183 | F17 | Healthy bulb | 12/22/2008 | *F. concentricum* | NJ, USA | HQ379640 | JN099742 |
| 54184 | F18 | Healthy bulb | 8/20/2008 | *F. proliferatum* | NJ, USA | HQ379688 | JN167216 |
| 54185 | F19 | Diseased bulb | 8/20/2008 | *F. solani* | NJ, USA | HQ379663 | JN167186 |
| 54186 | F20 | Healthy bulb | 3/9/2009 | *F. proliferatum* | NJ, USA | HQ379691 | JN167217 |
| 54187 | F21 | Healthy bulb | 4/1/2009 | *F. concentricum* | NJ, USA | HQ379647 | JN099743 |
| 54360 | F22 | Soil | 12/14/2009 | *F. proliferatum* | NJ, USA | HQ379695 | JN167218 |
| 54347 | F23 | Soil | 12/14/2009 | *F. concentricum* | NJ, USA | HQ379641 | JN099744 |
| 54349 | F24 | Soil | 12/14/2009 | *F. concentricum* | NJ, USA | HQ379642 | JN099745 |
| 54348 | F25 | Soil | 12/14/2009 | *F. concentricum* | NJ, USA | HQ379643 | JN099746 |
| 54359 | F26 | Soil | 12/14/2009 | *F. proliferatum* | NJ, USA | HQ379692 | JN167219 |
| 54358 | F27 | Soil | 12/14/2009 | *F. proliferatum* | NJ, USA | HQ379693 | JN167220 |
| 54350 | F28 | Soil | 12/14/2009 | *F. concentricum* | NJ, USA | HQ379644 | JN099747 |
| 54351 | F29 | Soil | 12/14/2009 | *F. concentricum* | NJ, USA | HQ379645 | JN099748 |
| 54357 | F30 | Soil | 12/14/2009 | *F. proliferatum* | NJ, USA | HQ379694 | JN167221 |
| 54343 | F31 | Soil | 12/14/2009 | *F. oxysporum* | NJ, USA | HQ379655 | JN167180 |
| 54344 | F32 | Soil | 12/14/2009 | *F. oxysporum* | NJ, USA | HQ379652 | JN167176 |
| 54352 | F33 | Soil | 12/14/2009 | *F. solani* | NJ, USA | HQ379665 | JN167187 |
| 54345 | F34 | Soil | 12/14/2009 | *F. oxysporum* | NJ, USA | HQ379659 | JN167181 |
| 54346 | F36 | Soil | 12/14/2009 | *F. oxysporum* | NJ, USA | HQ379660 | JN167181 |
| 54353 | F37 | Diseased bulb | 1/19/2010 | *F. solani* | OR, USA | HQ379669 | JN167209 |
| 54356 | F38 | Diseased bulb | 1/19/2010 | *F. solani* | OR, USA | HQ379670 | JN167208 |
| 54355 | F39 | Diseased bulb | 1/19/2010 | *F. solani* | OR, USA | HQ379671 | JN167194 |
| 54354 | F40 | Diseased bulb | 1/19/2010 | *F. solani* | OR, USA | HQ379672 | JN167207 |
| 54388 | F41 | Diseased bulb | 1/19/2010 | *F. solani* | OR, USA | HQ379685 | JN167206 |
| 54389 | F42 | Diseased bulb | 1/19/2010 | *F. solani* | OR, USA | HQ379682 | JN167189 |
| 54390 | F43 | Soil | 1/20/2010 | *F. solani* | OR, USA | HQ379673 | JN167203 |
| 54391 | F44 | Soil | 1/20/2010 | *F. solani* | OR, USA | HQ379664 | JN167204 |

**Table 1** (continued)

| [a]NRRL | Isolate No | Source | Date isolated | ID | Geographic origin | ITS GenBank accession no | TEF GenBank accession no |
|---|---|---|---|---|---|---|---|
| 54392 | F45 | Soil | 1/20/2010 | *F. solani* | OR, USA | HQ379684 | JN167193 |
| 54393 | F46 | Soil | 1/20/2010 | *F. solani* | OR, USA | HQ379683 | JN167203 |
| 54394 | F47 | Soil | 1/20/2010 | *F. oxysporum* | OR, USA | HQ379650 | JN167177 |
| 54395 | F48 | Soil | 1/20/2010 | *F. oxysporum* | OR, USA | HQ379658 | JN167183 |
| 54396 | F49 | Soil | 1/20/2010 | *F. avenaceum* | OR, USA | HQ379699 | JN167211 |
| 54397 | F50 | Soil | 1/20/2010 | *F. solani* | OR, USA | HQ379686 | JN167190 |
| 54398 | F51 | Soil | 1/20/2010 | *F. oxysporum* | OR, USA | HQ379654 | JN167174 |
| 54399 | F52 | Healthy bulb | 1/21/2010 | *F. solani* | OR, USA | HQ379674 | JN167202 |
| 54400 | F53 | Healthy bulb | 1/21/2010 | *F. solani* | OR, USA | HQ379680 | JN167191 |
| 54401 | F54 | Healthy bulb | 1/21/2010 | *F. solani* | OR, USA | HQ379675 | JN167201 |
| 54402 | F55 | Healthy bulb | 1/21/2010 | *F. oxysporum* | OR, USA | HQ379651 | JN167173 |
| 54491 | F56 | Healthy bulb | 1/21/2010 | *F. solani* | OR, USA | HQ379676 | JN167200 |
| 54492 | F57 | Healthy bulb | 1/21/2010 | *F. oxysporum* | OR, USA | HQ379657 | JN167184 |
| 54493 | F58 | Healthy bulb | 1/21/2010 | *F. solani* | OR, USA | HQ379677 | JN167210 |
| 54494 | F59 | Healthy bulb | 1/21/2010 | *F. oxysporum* | OR, USA | HQ379653 | JN167172 |
| 54495 | F60 | Healthy bulb | 1/21/2010 | *F. solani* | OR, USA | HQ379667 | JN167199 |
| 54496 | F61 | Healthy bulb | 1/21/2010 | *F. solani* | OR, USA | HQ379679 | JN167198 |
| 54497 | F62 | Healthy bulb | 1/21/2010 | [b]*Cylindrocarpon* sp. | OR, USA | HQ379698 | JN106065 |
| 54498 | F63 | Healthy bulb | 1/21/2010 | [b]*Cylindrocarpon* sp. | OR, USA | HQ379697 | JN106066 |
| 54499 | F64 | Healthy bulb | 1/21/2010 | *F. avenaceum* | OR, USA | HQ379700 | JN167212 |
| 54500 | F65 | Healthy bulb | 1/21/2010 | *F. solani* | OR, USA | HQ379668 | JN167197 |
| 54501 | F66 | Healthy bulb | 1/21/2010 | *F. solani* | OR, USA | HQ379666 | JN167196 |
| 54502 | F67 | Healthy bulb | 1/21/2010 | *F. solani* | OR, USA | HQ379681 | JN167192 |
| 54503 | F68 | Healthy bulb | 1/21/2010 | *F. solani* | OR, USA | HQ379681 | JN167195 |
| 54504 | F71 | Healthy bulb | 1/21/2010 | [b]*Cylindrocarpon* sp. | OR, USA | HQ379678 | JN106064 |
| *25181 | | | | *F. concentricum* | | U61678 | AF160282 |
| *22400 | | | | *F. solani* | | DQ094303 | AF178343 |
| *22395 | | | | *F. solani* | | AF178405 | AF178341 |
| *31071 | | *Triticum aestivum* | | *F. proliferatum* | | AF291061 | AF291058 |
| *43499 | | | | *F. oxysporum* | | DQ790539 | DQ790495 |
| *43542 | | | | *F. oxysporum* | | DQ790553 | DQ790509 |
| | *0109CI24X1 | Rodent excrement | | *F. avenaceum* | Spain | FN598933 | |
| | *FRC R-9496 | *Lisianthus* | | *F. avenaceum* | CA, USA | | EU357824 |
| | *CBS102032 | Bark of grapevine | | *Cylindrocarpon* sp. | Venezuela | AM419059 | |
| | *TBT-1 | Tail of black snail Tortoise | | *Cylindrocarpon* sp. | Japan | | AB373728 |

*ITS and TEF Sequence information obtained from GenBank and included in this study to represent known species of fusaria and *Cylindrocarpon* sp.

[a] NRRL = USDA Agricultural research service (ARS) culture collection

[b] *Cylindrocarpon* strains isolated from lily bulbs and used in the phylogenetic study as outgroup species

(Nixon 1999; Goloboff 1999). All characters were weighted equally and alignment gaps were treated as missing data. We also conducted bootstrap analyses to estimate the confidence of branching points. The Consistency Index (CI) and Retention Index (RI) were calculated to indicate the amount of homoplasy present. *Cylindrocarpon* isolates were used as an outgroup.

### Mating populations (MPs)

The GFSC isolates were crossed on carrot agar with tester strains of MPs A, B, D, E and F from the Fungal Genetic Stock Center (Kansas City, MO, USA), with the mycelial plug method as described by Leslie and Summerell (2006). Crosses were deemed positive when mature purple perithecia were observed with oozing ascospores of normal morphology. The crossing experiment was conducted twice.

### In vitro toxin analysis

The GFSC isolates were evaluated for their in vitro toxin production. Rice kernels (100 g, 45% moisture content) were kept overnight in 500 ml Erlenmeyer flasks at room temperature, then autoclaved for 20 min at 121°C. Autoclaved rice was inoculated with 2 ml of an aqueous suspension containing $10^6$ spore $ml^{-1}$ of each isolate, two flasks for each isolate. Flasks were incubated in the dark at 25°C for 4 weeks. At the end of incubation time, harvested cultures were dried in a forced-draft oven at 60°C for 48 h, finely ground and stored at 4°C until use. Uninoculated kernels were used as controls.

### Chemicals and reagents

All solvents (acetonitrile, methanol, formic acid) of HPLC grade were purchased from Merck (Whitehouse Station, NJ, USA.). Deionized water (< 8MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath. The stock standard solution of beauvericin (BEA) was purchased from Sigma-Aldrich (St. Louis, MO, USA.). Standard solutions of fusaproliferin (FUS) and enniatins (ENs: ENA, $ENA_1$, ENB and $ENB_1$) were kindly provided by Professor Alberto Ritieni, Department of Food Science, University of Naples "Federico II", Italy. All stock solutions were prepared by dissolving 1 mg of the mycotoxin (BEA, FUS or ENs) in 1 ml of pure methanol, obtaining a 1 mg $ml^{-1}$ solution. These stock solutions were then diluted with pure methanol in order to obtain the appropriated working solutions. All solutions were stored in darkness at 4°C until HPLC analysis. Fumonisins $B_1$ ($FB_1$) and $B_2$ ($FB_2$) standards were provided by Sigma-Aldrich (Madrid, Spain); fumonisin $B_3$ ($FB_3$) was supplied by PROMEC (Programme on Mycotoxins and Experimental carcinogenesis, Tygerbei, Republic of South Africa). Stock standard solutions of the three toxins were up to 98% of purity and were prepared by dissolving 1 mg of each fumonisin in 1 ml of pure acetonitrile-water (50:50 v/v), obtaining 1 mg $ml^{-1}$ of each solution. These stock solutions were then diluted with pure acetonitrile-water (50:50 v/v) in order to obtain the appropriated working solutions.

### BEA, ENs and FUS extraction procedure

Analysis of BEA, ENs and FUS followed Jestoi's (2008) methods. Briefly, mycelium samples (3 g) were extracted with 20 ml of methanol using an Ika T18 basic Ultraturrax (Staufen, Germany) for 3 min. The extract was centrifuged at 4,500 g for 5 min and then the supernatant evaporated to dryness with a Büchi Rotavapor R-200 (Postfach, Switzerland) and then re-dissolved in 2 ml of extraction solvent. This final solution was filtered through a 25 mm/0.22 μm nylon filter purchased from Análisis Vínicos (Tomelloso, Spain) before injection into the LC-DAD system for analysis.

### LC-DAD analysis

LC analyses of BEA, ENs and FUS were performed using a Shimadzu LC system equipped with LC-10 AD pumps and a diode array detector (DAD) from Shimadzu (Japan). A Gemini (150×4.6 mm, 5 μm) Phenomenex column was used. HPLC conditions were set up using a constant flow at 1.0 ml $min^{-1}$ and acetonitrile-water (70:30 v/v) as starting diluent solvent. The starting ratio was kept constant for 5 min and then linearly modified to 90% acetonitrile in 10 min. After 1 min at 90% acetonitrile, the mobile phase was taken back to the starting conditions in 4 min. BEA and ENs were detected at 205 nm, while FUS was detected at 261 nm. All samples were filtered through a 0.22 μm syringe filter (Phenomenex) prior to injection (20 μl) onto the column. Mycotoxin identification was performed by comparing retention times and UV spectra of purified extracted samples to pure standards. Quantification of mycotoxins was carried out by comparing peak areas of investigated samples to the calibration curve of authentic standards.

### Method validation

Mean recoveries using fortified cereal samples ($n$=3) at levels of $EN_S$ (0.3–50 μg $g^{-1}$), FUS (0.3–50 μg $g^{-1}$) and BEA (0.3–50 μg $g^{-1}$) were 84.6, 70.5, and 88.6% with coefficients of variation being 3.5, 4.6 and 3.2%, respectively. Intra-day ($n$=5) and inter-day (5 different days) variation values at were 2.5–8.6, 2.0–10.4 and 2.5–8.3%, respectively for ENs, FUS and BEA. These values are below 15%, which is the maximum variation for certification exercises for several mycotoxins. The limit of detection (LOD) and quantification (LOQ) calculated according to a signal/noise (s/n)=3 and 10, respectively, are 0.21 and 0.60 mg $kg^{-1}$ for FUS, 0.17 and 0.50 mg $kg^{-1}$ for BEA, and 0.215 and 0.60 mg $kg^{-1}$ for ENA, 0.14 and 0.40 mg $kg^{-1}$ for $ENA_1$, 0.145 and 0.40 mg $kg^{-1}$ for ENB and finally 0.165 and 0.50 mg $kg^{-1}$ for $ENB_1$.

### Fumonisin analyses

A modified method of Chelkowski et al. (2007) was carried out for fumonisin extractions. Fifty grams of dried samples were homogenized by shaking with 500 ml of methanol-water (70:30 v/v) with Ika T18 basic Ultraturrax (Staufen, Germany) for 5 min. Samples were then filtered through Phenomenex no. 4 filter paper, and the solvent was removed under reduced pressure. The extract was dissolved in 50 ml of methanol-water (70:30 v/v) and filtered through a paper filter Phenomenex before toxin identification and quantification by liquid chromatography (LC) as described by D'Arco et al. (2008).

### LC-MS/MS analysis of fumonisins

LC separation was carried out on a Luna $C_{18}$ analytical column (150 mm×4.6 mm I.D., 5 μm) preceded by a $C_{18}$ security guard cartridge (4 mm×2 mm I.D., 5 μm), both from Phenomenex (Madrid, Spain). The analytical separation was performed using gradient elution with water as mobile phase A and methanol as mobile phase B, both containing 0.5% formic acid. After an isocratic step of 65% B for 3 min, it was gradually increased to 95% B in 4 min and held constantly for 3 min. Afterwards, the initial conditions were maintained for 10 min. Flow rate was maintained at 0.3 ml $min^{-1}$. A TQ mass spectrometer Quattro LC from Micromass (Manchester, U.K.), equipped with an LC Alliance 2695 system (Waters, Milford, MA), consisted of an auto-sampler, a quaternary pump, a pneumatically assisted electrospray probe, a Z-spray interface, and a Mass Lynx NT software 4.1 was used for data acquisition and processing. Parameters were optimized by continuous infusion of a standard solution (10 μg $ml^{-1}$) via a syringe pump at a flow rate of 10 μl $min^{-1}$. The analysis was performed in positive ion mode. The ESI source values were as follows: capillary voltage, 3.20 kV; cone, 50 V; extractor, 3 V; RF lens, 0.2 V; source temperature, 125°C; desolvation temperature, 300°C; desolvation gas (nitrogen, 99.99% purity) flow, 500 l $h^{-1}$; cone (gas flow) 50 l $h^{-1}$. The analyzer settings were: resolution, 12.0 (unit resolution) for the first and third quadrupoles; ion energy, 0.5; entrance and exit energies, −3 and 1; multiplier, 650 V; collision gas (argon, 99.995%) pressure $3.74\times10^{-3}$ mbar; interchannel delay, 0.02 s; total scan time, 1.0 s. The mass spectrometer was operated in scan, product ion scan, and multiple reaction monitoring (MRM) modes. All the measurements were carried out by triplicate.

### Method validation

Precision was calculated in terms of intra-day repeatability ($n$=5) and inter-day reproducibility (5 different days) on a standard of 0.5 μg $ml^{-1}$. The intra-day repeatability led to relative standard deviation (RSD) values that ranged from 1.5% to 6.1%. The inter-day reproducibility was lower than 9.4% for all instances. The established protocol, LC–(ESI)-MS/MS, was validated by the analysis of spiked samples. At 20 μg $kg^{-1}$, recoveries ranged from 82% for $FB_1$ to 68% for $FB_2$ with RSDs below 12%. The LOD was estimated for those concentrations that provide a signal to noise ratio of 1:3. These values of the LODs are 0.7 μg $kg^{-1}$ for the $FB_1$ and $FB_2$ and 1.5 μg $kg^{-1}$ for $FB_3$. LOQs estimated as those concentrations of analyte which yield a signal-to-noise of at least 1:10, range from 2 μg $kg^{-1}$ for $FB_1$ and $FB_2$ and of 5 μg $kg^{-1}$ for $FB_3$.

## Results

### Identification of fungi

A total of 35 *Fusarium* isolates were obtained from the NJ site and 30 from the OR site. Based on the morphological and molecular identification, the most

commonly isolated *Fusarium* species from the NJ site was *F. concentricum* (43%), followed by *F. proliferatum* (26%), *F. oxysporum* (20%) and *F. solani* (11.0%). The most frequently isolated species from the OR site was *F. solani* (74.0%), followed by *F. oxysporum* (20%) and *F. avenaceum* (6%). Three *Cylindrocarpon* isolates (anamorphic state of *Neonectria*) were also isolated from the OR samples (healthy bulbs) and were used as an outgroup in the phylogenetic analyses. *Fusarium solani* and *F. oxysporum* were isolated from both NJ and OR sites. However, *F. concentricum* and *F. proliferatum* were only found from the soil and healthy bulbs in the NJ site, while *F. avenaceum* was isolated only from the OR site.

### Mating population and mating type study

Out of the 65 isolates of fusaria, 24 belong to GFSC (*F. proliferatum* and *F. concentricum* clades, Fig. 1). All of the *F. proliferatum* isolates produced purple perithecia when crossed with the *MATD-1* tester strain on carrot agar. The results of the diagnostic multiplex assay also confirmed that all nine *F. proliferatum* isolates were *MAT-2*. PCR assay indicated that the 15 *F. concentricum* isolates had the *MAT-2* gene, but they did not produce perithecia with any of the tester strains.

### Toxin analysis

All the GFSC isolates tested were positive for the presence of the enniatin gene following the PCR based assay. The results of toxin production by isolates of *F. proliferatum* and *F. concentricum* grown on rice grains are summarized in Table 2. All the isolates, except two isolates of *F. proliferatum* (F18 and F22) and one isolate of *F. concentricum* (F24) produced BEA in concentrations ranging from 31.41 to 302.51 μg $g^{-1}$ dry weight of rice. ENA was produced by one isolate of *F. proliferatum* (4.93 μg $g^{-1}$). ENB was produced by two *F. concentricum* strains (F13 and F14) and also two *F. proliferatum* strains (F16 and F18) at concentrations ranging from 1.29 to 14.7 μg $g^{-1}$. $ENB_1$ was produced by three *F. concentricum* strains (F9, F10 and F14) and two *F. proliferatum* strains (F8 and F20) at concentrations ranging from 7.44 to 12.26 μg $g^{-1}$. $ENA_1$ and fumonisins B1, B2 and B3 could not be detected from any of the isolates.

### Phylogenetic analyses

DNA sequences generated in this study were deposited in Genbank under accession numbers: HQ379633-HQ379700 (ITS) and JN099734 - JN099748, JN106064-JN106066, JN167172-JN167221 (TEF). The aligned sequences were deposited in TreeBASE (http://www.treebase.org) under submission id 11175. The combined ITS and TEF dataset contained 76 taxa and 1,088 characters, among which 432 were parsimony informative. Eight ITS and eight TEF sequences from previously identified isolates of *F. concentricum, F. solani, F. oxysporum, F. proliferatum, F. avenaceum* and *Cylindrocarpon* sp. from GenBank were also included in the analyses (Table 1). Parsimony analysis for the combined data yielded 80 most parsimonious trees of 822 steps (CI=0.78, RI=0.97). Based on the phylogeny, the ingroup taxa divided into five groups, namely *F. oxysporum, F. solani, F. avenaceum, F. concentricum,* and *F. proliferatum* with high bootstrap and Bayesian posterior probability support (Fig. 1). The trees derived from Bayesian and parsimony analysis were topologically similar (Fig. 1). The individual gene phylogenies were similar to the combined tree, except that the ITS tree did not separate *F. concentricum* and *F. proliferatum*.

## Discussion

The main objective of this study was to identify and characterize the *Fusarium* species associated with *L. longiflorum.* Analysis of fusaria in *L. longiflorum* from two ecologically distinct sites yielded quite different species composition. The OR site has long been used for lily bulb cultivation while the NJ site has had no history of bulb cultivation until now. The dominant species of the NJ site were *F. concentricum* and *F. proliferatum*, both members of GFSC. At the OR site, *F. solani* dominated. *F. solani* and *F. oxysporum* were present in both locations, but *F. avenaceum* was observed only in the samples from OR. The differences in fusarium diversity at the two sites reflect their different agricultural histories and disease pressure. The OR site has been in continual lily bulb cultivation for decades with bulb production every 4 years. Fungicides must be applied to control diseases (Lee Riddle, Personal communication). *Fusarium oxysporum* and *F. solani* are well known

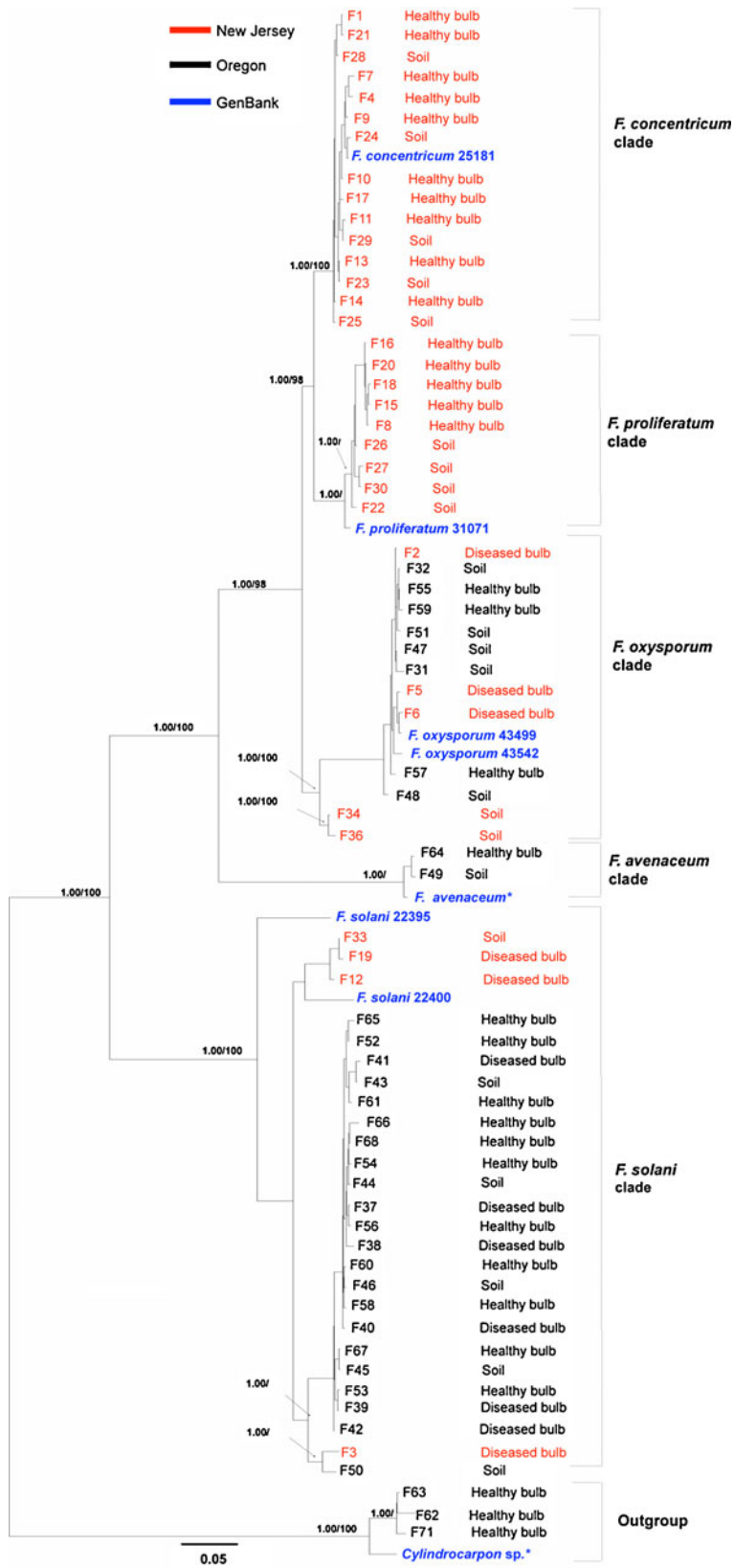


**Fig. 1** Bayesian inference tree based on the combined DNA sequences of partial internal transcribed spacer region (ITS) and translation elongation factor1-α gene (TEF). Phylogenetic analysis was performed on 65 *Fusarium* isolates, 3 *Cylindrocarpon* isolates (outgroup) obtained in this study, and 8 reference sequences selected from GenBank. Posterior probabilities (PP) and parsimony bootstrap percentages (BS) are shown as PP/BS on branches that have ≥0.95 PP or ≥80% BS support. *Cylindrocarpon* species were selected as outgroup to root the tree. * Indicates that ITS and TEF sequence data came from two different isolates

**Table 2** In vitro toxin production by *F. proliferatum* and *F. concentricum* from Easter lilies

| Isolate No[a] | *Fusarium* species | BEA[b] (μg $g^{-1}$) | ENB (μg $g^{-1}$) | $ENB_1$ (μg $g^{-1}$) | ENA (μg $g^{-1}$) | FUS (μg $g^{-1}$) |
|---|---|---|---|---|---|---|
| F1 | *F. concentricum* | 198.42 | nd | nd | nd | nd |
| F4 | *F. concentricum* | 209.32 | nd | nd | nd | nd |
| F7 | *F. concentricum* | 214.66 | nd | nd | nd | nd |
| F9 | *F. concentricum* | 113.41 | nd | 11.46 | nd | nd |
| F10 | *F. concentricum* | 160.57 | nd | 8.9 | nd | nd |
| F11 | *F. concentricum* | 127.21 | nd | nd | nd | nd |
| F13 | *F. concentricum* | 31.41 | 3.15 | nd | nd | nd |
| F14 | *F. concentricum* | 182.04 | 1.29 | 11.67 | nd | nd |
| F17 | *F. concentricum* | 60.41 | nd | nd | nd | nd |
| F21 | *F. concentricum* | 134.59 | nd | nd | nd | nd |
| F23 | *F. concentricum* | 45.16 | nd | nd | nd | nd |
| F24 | *F. concentricum* | nd | nd | nd | nd | nd |
| F25 | *F. concentricum* | 302.51 | nd | nd | nd | nd |
| F28 | *F. concentricum* | 184.11 | nd | nd | nd | nd |
| F29 | *F. concentricum* | 92.62 | nd | nd | nd | nd |
| F8 | *F. proliferatum* | 75.79 | nd | 7.44 | nd | 3.06 |
| F15 | *F. proliferatum* | 39.40 | nd | nd | nd | nd |
| F16 | *F. proliferatum* | 42.85 | 8.59 | nd | nd | nd |
| F18 | *F. proliferatum* | nd | 14.78 | nd | nd | nd |
| F20 | *F. proliferatum* | 87.93 | nd | 12.26 | nd | 1.37 |
| F22 | *F. proliferatum* | nd | nd | nd | 4.93 | nd |
| F26 | *F. proliferatum* | 162.15 | nd | nd | nd | 3.60 |
| F27 | *F. proliferatum* | 96.19 | nd | nd | nd | 1.73 |
| F30 | *F. proliferatum* | 100.43 | nd | nd | nd | 1.48 |

[a] Isolates grown on autoclaved rice kernels in the dark at 25°C for 4 weeks. BEA = beavericin, FUP = fusaproliferin, EN = Enniatin, nd = not detected

[b] Detection limits: $FB_1$, $FB_2$=0.7 μg $g^{-1}$, $FB_3$=1.5 μg $g^{-1}$, Fumonisins undetected. FUS=0.21 μg $g^{-1}$ BEA=0.17 μg $g^{-1}$, ENA= 0.215 μg $g^{-1}$, $ENA_1$=0.14 μg $g^{-1}$, ENB=0.145 μg $g^{-1}$, and $ENB_1$=0.165 μg $g^{-1}$

pathogens of lilies and their frequent occurrence in the OR site can be explained by the presence of a suitable host. On the other hand, at the NJ site no history of commercial bulb production and therefore low disease pressure makes fungicide use unnecessary and allows for a greater diversity of fusaria including many endophytic types that may compete with pathogens.

The *Fusarium* populations identified from the OR region are similar to previous studies of the pathogens associated with Easter lilies (Bald and Solberg 1960). However, the predominant presence of *F. proliferatum* and *F. concentricum* from healthy bulbs and soil in NJ has not been reported. This result could also be explained by the crop history of the NJ site. The NJ fields were previously planted with asparagus, also in the Liliaceae and highly susceptible to infection by *F. proliferatum* (Elmer et al. 1996) among other *Fusarium* species. Less pathogenic strains may have persisted in the soil and built up over a period of time. They would gradually gain entry into the bulbs. These milder strains usually cause a yellowing in the epidermal layers, although never any signs of rot (Bald et al. 1983).

*Fusarium avenaceum* is usually associated with cereal grains, and was also isolated from crop soil samples grown in a temperate climate (Leslie and Summerell 2006). Although there have been a few reports of *F. proliferatum* associated with lilies, the isolation of *F. proliferatum* and pathogenicity of this species on Easter lilies has never been studied.

*Fusarium concentricum* has also not previously been reported to be associated with Easter lilies. To our knowledge, this is the first report of the isolation and characterization of *F. concentricum* from Liliaceae. Predominantly isolated from *Musa x paradisiaca* (banana) in Central America and *Nilaparvata lugens* (Asian brown leaf hopper) in Korea (Nirenberg and O'Donnell 1998), *F. concentricum* was described as a new species by Nirenberg and O'Donnell (1998). Morphologically similar to *F. circinatum*, *F. guttiforme* and other *Fusarium* species, it was likely misidentified even if it has been isolated from *Lilium* or other hosts previously. It is a poorly described species within the GFSC and produces beauvericin, but there are no reports of the production of other mycotoxins (Fotso et al. 2002). Very little is known about the pathogenicity and biology of *F. concentricum* (Leslie and Summerell 2006).

In contrast to *F. solani* and *F. oxysporum*, which were isolated from soil, as well as healthy and rotting bulbs, the GFSC species were always isolated from soil or only healthy, symptomless bulbs, indicating their endophytic association with lilies. There is now considerable evidence to support the establishment of fusarial endophytic colonization in many plants. The generally accepted definition of endophyte has also broadened now, to include organisms that at some point in their life cycle, establish a symptomless infection of internal tissues of a plant (Petrini 1991).

All of the *F. proliferatum* isolates in this study are *MAT-2* and belong to MP D. *F. concentricum* did not produce perithecia with any MP testers. However, the results of the diagnostic multiplex assay showed that all of the 15 isolates of *F. concentricum* were also *MAT-2*. This suggests a lack of sexual reproduction among the isolates.

TEF is thought to be of great utility in fungal phylogenetic studies. This single copy protein-coding gene showed high level of sequence polymorphisms among closely related species in *Fusarium* (Geiser et al. 2004). In this study, TEF provided informative characters to separate *F. concentricum* and *F. proliferatum* isolates, which could not be distinguished with the ITS sequence alone.

Both BEA and ENs are cyclic hexadepsipeptides with insecticidal, antimicrobial, phytotoxic, and cytotoxic activities (Jestoi 2008). BEA was detected from the majority of the GFSC isolates used in this study within the range previously reported (Fotso et al. 2002). All of the GFSC isolates tested are potential enniatin producers based on the PCR detection results. However, only low amounts (<15 μg $g^{-1}$) of ENA, ENB, and $ENB_1$ were detected in a few isolates, indicating that the gene was not actively expressed under the culturing conditions used in this study. Interestingly, our *F. proliferatum* strains did not produce $FB_1$, $FB_2$ or $FB_3$. Fumonisins are a group of nongenotoxic carcinogens that are typically produced by *F. proliferatum*. These atypical non-producers obtained from Easter lily could be of significant importance in the biocontrol of *Fusarium* diseases. Biological control of fumonisin producing fusaria in maize by competitive exclusion occurred with a natural variant of *F. verticilloides* that produces undetectable level of fumonisins. This strategy relies on the ability of non-toxigenic strains to effectively compete with mycotoxin producers in the agricultural environment (Desjardins and Plattner 2000). Fumonisins are phytotoxic and reduce shoot and root growth. Although non-FB producing strains may still be able to cause disease under some circumstances, the lack of fumonisin production by the GFSC isolates could also be one of the reasons that enable them to maintain an endophytic lifestyle. This study provides baseline information for further investigating the ecological role of *Fusarium* in plant-fungal interactions. Whether these endophytic isolates offer any protection against pathogenic *Fusarium* isolates is a question we hope to answer in ongoing and subsequent studies.

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