Fumonisin and Beauvericin Chemotypes and Genotypes of the Sister Species

*Fusarium subglutinans* and *Fusarium temperatum*

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ABSTRACT  *Fusarium subglutinans* and *F. temperatum* are common maize pathogens that produce mycotoxins and cause plant disease. The ability of these species to produce beauvericin and fumonisins is not settled, as reports of toxin production are not concordant. Our objective was to clarify this situation by determining both chemotypes and genotypes for strains from both species. We analyzed 25 strains from Argentina – 13 of *F. subglutinans* and 12 of *F. temperatum* – for toxin production by UPLC-MS. We used new genome sequences from two strains of *F. subglutinans* and one strain of *F. temperatum*, plus genomes of other *Fusarium* species, to determine the presence of functional gene clusters for the synthesis of these toxins. None of the strains examined from either species produced fumonisins. These strains also lack *Fum* biosynthetic genes, but retain homologs of some genes that flank the *Fum* cluster in *F. verticillioides*. None of the *F. subglutinans* strains we examined produced beauvericin, although nine of 12 *F. temperatum* strains did. A complete beauvericin (*Bea*) gene cluster was present in all three new genome sequences. The *Bea1* gene was presumably functional in *F. temperatum*, but was not functional in *F. subglutinans* due to a large insertion and multiple mutations that resulted in premature stop codons. The accumulation of only a few mutations expected to disrupt *Bea1* suggests that the process of its inactivation is relatively recent. Thus, none of the
strains of *F. subglutinans* or *F. temperatum* we examined produce fumonisins and the strains of
*F. subglutinans* examined cannot produce beauvericin either. Variation in the ability of strains of
*F. temperatum* to produce beauvericin requires further study and could reflect the recent shared
ancestry of these two species.

**IMPORTANCE** *Fusarium subglutinans* and *F. temperatum* are sister species and maize
pathogens commonly isolated worldwide, which can produce several mycotoxins and cause
seedling disease, stalk rot and ear rot. The ability of these species to produce beauvericin and
fumonisin mycotoxins is not settled, as reports of toxin production are not concordant at the
species level. Our results are consistent with previous reports that strains of *F. subglutinans*
produce neither fumonisins nor beauvericin. The status of toxin production by *F. temperatum*
still needs further work. Our strains of *F. temperatum* did not produce fumonisins, while some
strains produced beauvericin and others did not. These results enable more accurate risk
assessments of potential mycotoxin contamination if strains of these species are present. The
nature of the genetic inactivation of BEA1 is consistent with its relatively recent occurrence and
the close phylogenetic relationship of the two sister species.

**KEYWORDS** comparative genomics, gene inactivation, maize pathogens, mycotoxin
biosynthesis, secondary metabolism
Fusarium subglutinans is an important pathogen of maize commonly isolated worldwide, and considered a causal agent of seedling disease, stalk rot and ear rot (1). This species also can produce a broad range of mycotoxins (2). Within the morphological F. subglutinans sensu lato species, two populations were identified based on DNA sequence data (3). The two populations, F. subglutinans Group 1 and F. subglutinans Group 2, appeared to be reproductively isolated in nature and were presumed to be in the process of sympatric genetic divergence (3). Fusarium subglutinans Group 1 has now been formally described as Fusarium temperatum (4), while F. subglutinans Group 2 has retained the formal Fusarium subglutinans sensu stricto name.

Mycotoxin production by these species is of particular interest because production of beauvericin, a cyclic hexadepsipeptide with insecticidal and carcinogenic properties (5-7), has been reliably reported only in F. temperatum (Group 1) and not in F. subglutinans (8-11). Beauvericin production has been used to identify the species to which some strains belong (11).

Continuing studies of F. temperatum and F. subglutinans on cereals, primarily maize (12-20) have resulted in a general consensus that beauvericin is produced only by strains of F. temperatum and not by strains of F. subglutinans, but the genetics underlying these differences has not been investigated in any detail. Differences in beauvericin production by these two closely related species could provide insights into the evolutionary processes involved in their separation into different species.

The beauvericin (Bea) biosynthetic gene cluster was first described in F. fujikuroi IMI 58289, and consists of a four-gene cluster: Bea1, which encodes the NRPS22, the non-ribosomal peptide synthase responsible for synthesizing the beauvericin backbone, and Bea2, Bea3 and Bea4, which encode proteins with transport and regulatory functions (21). Orthologous four-gene
biosynthetic clusters also are known in *F. proliferatum*, *F. mangiferae*, and *F. oxysporum* (21), all of which are reported as beauvericin producers in multiple studies (22, 23). *Fusarium proliferatum* is a common contaminant of cereals such as maize, wheat and barley, and can contaminate these substrates with beauvericin as well (22). *Fusarium mangiferae* is a major cause of mango malformation worldwide (24), but a role for beauvericin in its phytotoxicity has not yet been identified. In *F. oxysporum*, a causal agent of tomato wilt, beauvericin reduces the level of ascorbic acid in the tomato cells leading to the collapse of the ascorbate system and protoplast death (25).

The fumonisin (*Fum*) biosynthetic gene cluster in the genus *Fusarium* has been well described, and includes 16 genes that encode biosynthetic enzymes and regulatory and transport proteins. Functions of genes in fumonisin biosynthesis have been determined in *F. verticillioides* (26), and the number, order and genomic orientation of the *Fum* genes are known in *F. proliferatum* and *F. oxysporum* (27-29). Sequences flanking the *Fum* gene cluster differ among species, however, indicating that the cluster’s genomic location is species dependent (26). Reports of fumonisin production on cracked corn (10, 14, 20, 30, 31) by some strains of *F. temperatum* and *F. subglutinans* are inconsistent with reported genetic capabilities for fumonisin biosynthesis by these species, as sequenced strains of both *F. subglutinans* and *F. temperatum* lack one or more of the *Fum* genes required for fumonisin biosynthesis (26, 28, 32).

The objectives of this study were to further test the ability of these species to synthesize beauvericin and/or fumonisin with definitive chemical tests of strains not cultured on cracked corn and genetic analyses of additional strains. Our working hypotheses were: (i) That no strains of either species could synthesize fumonisin; (ii) that *F. temperatum* strains, but not those of *F. subglutinans*, could synthesize beauvericin, and (iii) that the chemical phenotypes would be
consistent with the genomic sequence genotypes. The study advances the field by providing new insights into the toxigenic potential of these species and enabling more accurate estimation of the risks they pose to the food and feed products they might contaminate.

RESULTS

Strain Isolation and Identification

Twenty-five *Fusarium* strains from Argentina (Table 1) were identified to species in a ML phylogenetic analysis of a 3-gene combined data set, including sequences of reference strains from related species, with *F. proliferatum* NRRL 62905 as the outgroup (Fig. 1). Twelve strains were contained within a well-supported clade (bootstrap value: 88) that included the *F. temperatum* reference strain ITEM 16196 (MUCL 52463) (4). The remaining 13 strains were contained within a second well-defined clade (bootstrap value: 99) that included the *F. subglutinans* reference strain NRRL 22016 (Fig. 1).

Genome Analyses

**Genome assemblies.** We generated genome assemblies for two strains of *F. subglutinans* (RC 298 and RC 528) and one strain of *F. temperatum* (RC 2914) (Table 2). For *F. temperatum* RC 2914, ~7.7 million reads were assembled in 720 scaffolds for a total length of 42.5 Mb when including only scaffolds ≥ 10 kb in length. The scaffold N50, *i.e.*, the length of the shortest scaffold such that 50% of the assembly is found in scaffolds of this length or longer,
was 334 kb, and the longest scaffold was 1.5 Mb. The average coverage was 53×. Two scaffolds were retained for analysis of the Bea and Fum clusters.

For F. subglutinans RC 298, ~17 million reads were assembled in 4,088 scaffolds for a total length of 49.7 Mb when including only scaffolds ≥ 10 kb in length. The scaffold N50 was 228 kb, and the largest scaffold was 975 kb. The average coverage was 101×. Two scaffolds were retained for analysis of the Bea and Fum clusters. Finally, for F. subglutinans RC 528, ~9 million reads were assembled in 1,418 scaffolds for a total length of 43 Mb when including only scaffolds ≥ 10 kb in length. The scaffold N50 was 204 kb, and the largest scaffold was 997 kb. The average coverage was 61×. Again, two scaffolds were retained for analysis of the Bea and Fum clusters.

Genomic context of contigs containing the beauvericin and fumonisin clusters.

Dotplot analysis between chromosome 9 of F. fujikuroi IMI 58289 (Ffuj_Chr9), where a complete Bea cluster is located, and Scaffold 7 of F. temperatum CMWF 389 (Ftemp_Scaff7) identified sequences of almost the same length with complete synteny. Thus, Ftemp_Scaff7 probably is orthologous to chromosome 9 predicted for F. fujikuroi (Fig. 2). Dot plot analysis between chromosome 1 of F. verticillioides FGSC 7600 (Fv_Chr1), where the Fum cluster is located, and two scaffolds of F. temperatum CMWF 389, Scaffold 1 (Ftemp_Scaff1) and Scaffold 12 (Ftemp_Scaff12), had very good synteny. Thus, chromosome 1 of F. verticillioides is orthologous to Ftemp_Scaff1 and Ftemp_Scaff12 (Fig. 3).

Circos plot analysis with the complete Bea cluster from Ffuj_Chr9 and portions of Ftemp_Scaff7 and the three newly sequenced Bea-containing contigs shows that the Bea cluster is complete in both F. temperatum and F. subglutinans (Fig. 4A).
Circos plot analysis with the complete Fum cluster from *F. verticillioides* Chromosome 1 and portions of contigs from the three newly sequenced strains shows a gap in the synteny. Thus, *F. temperatum* and *F. subglutinans* both lack most of the genes normally found in this biosynthetic cluster (Fig. 4B).

**Beauvericin Cluster**

The entire Bea cluster (21) is present in the *F. subglutinans* and *F. temperatum* strains sequenced in the current study, as well as in several other closely related species that produce beauvericin, *e.g.*, *F. fujikuroi*, *F. mangiferae*, *F. nygamai*, *F. oxysporum*, and *F. proliferatum* (Fig. 5). In *F. circinatum* FSP 34, the Zn(II)$_2$Cys$_6$ transcription factor (FFUJ_09298), encoded by Bea4, is absent, and this gene also is missing in the other two *F. circinatum* genomes in GenBank (strains GL 1327 and KS 17).

Complete and functional BEA2, BEA3 and BEA4 proteins are predicted for all three genomes assembled in this study. The Bea1 gene encoding the non-ribosomal peptide synthase NRPS22 is predicted to produce a functional protein in both *F. temperatum* strains (RC 2914 and CMWF 389). In *F. subglutinans*, the predicted protein is apparently nonfunctional in strain RC 528 due to a Single Nucleotide Polymorphism (SNP) resulting in a premature stop codon (CAG $\rightarrow$ TAG transition). This transition occurs at nucleotide position 7,685 (relative to the Bea1 sequence from *F. fujikuroi* IMI 58289), where position 1 coincides with the start of the reading frame, i.e., the adenine of the ATG start codon. In strain RC 298, there is an insertion of a single cytosine at position 5,875 that results in a frameshift and premature truncation of the protein (Fig. 6).
Both of the *F. subglutinans* strains had a 184 bp insertion between nucleotides 4,223 and 4,416 (Fig. 6). If this insertion was transcribed, it would add 61 amino acids to the length of the protein and cause a frameshift in the downstream reading frame that would lead to premature truncation of the protein. *In silico* prediction programs exclude the 184 bp insertion region from the open reading frame, and instead introduce novel introns to prevent the premature truncation of the protein due to in-frame stop codons within the insertion. This predicted gene transcript would still result in a large protein, but it is uncertain whether the resulting protein would function properly.

**Fumonisin Cluster**

The entire *Fum* cluster was missing from the *F. subglutinans* and *F. temperatum* genomes, which is consistent with the reported inability of many strains of these species to produce fumonisins. We searched for portions of all 16 *Fum* cluster genes (26), but found no recognizable homologous sequences. *Fusarium subglutinans* and *F. temperatum* are members of the American clade of the *F. fujikuroi* species complex (FFSC). Some members of this clade, e.g., *F. anthophilum* and *F. bulbicola*, can produce fumonisins and carry the *Fum* biosynthetic gene cluster (26, 32, 33). We queried our newly generated genomes and those of some other members of the FFSC with genes that flank the *Fum* cluster in species from all three clades of the FFSC (26). In all cases, the *Fum* cluster was absent from *F. subglutinans* and *F. temperatum*. Instead, we found one of four flanking genes (*Cpm2*) from the American clade species and two of four genes from Asian clade species (*Mfs1* and *Zcb1*). We also found all four genes queried from African clade species (*Znf1*,...
Zbd1, Orf20, and Orf21), though the orientation and order of Orf21 and Znf1 were different in F. subglutinans and F. temperatum with respect to F. verticillioides (Fig. 7).

### Mycotoxin Production

Nine of the 12 strains identified as F. temperatum produced beauvericin at levels ranging from 7 to 400 μg/kg (mean: 71 μg/kg; median: 11.3 μg/kg), whereas no F. subglutinans strains produced beauvericin. None of the 25 strains examined produced FB1 on PDA (Table 1).

### DISCUSSION

*Fusarium subglutinans* and *F. temperatum* are well known as pre-harvest fungal pathogens that cause maize stalk and ear rot, and are closely related species that can be easily misidentified as the other (4). Strains of these species can produce a variety of mycotoxins (2, 8, 10, 14, 30, 31, 34). However, reports of mycotoxin production by these species are not consistent, e.g., 14, 20, leading to confusion regarding the specific mycotoxin profile that they possess. This confusion can result in underestimation or overestimation of the mycotoxin associated risk posed by foods and feeds contaminated with these fungi. It also makes it very difficult to develop effective pre- and post-harvest strategies for monitoring and managing mycotoxin contamination.

There are multiple reports of fumonisin production (10, 14, 30, 31) and non-production (2, 14, 34, 35) by *F. subglutinans* Groups 1 and 2, which are now *F. temperatum* and *F. subglutinans*. The lack of all or parts of the *Fum* gene cluster in some strains of both species has been reported on multiple occasions (26, 27, 32). In our study, we found that some genomes of both species lacked the entire *Fum* cluster, and that the insertion sites across species in the FFSC
that contain part or all of the *Fum* gene cluster are not well conserved. For example, in *Fusarium musae*, a sister species of *F. verticillioides* that cannot produce fumonisins (36, 37) only remnants of the *Fum21* and *Fum19* genes, at the opposite ends of the cluster, remain along with some of the flanking genes. The deletions and rearrangements we detected in genomic regions where the *Fum* cluster is inserted in other species suggests that changes related to *Fum* cluster insertion/deletion are not simple events and could have occurred in more than one step at more than one time.

In contrast with fumonisins, there is a general consensus that strains of *F. subglutinans* do not produce beauvericin but that some strains of *F. temperatum* do (10-12, 14-16, 18). In the present study, we found that 75% of the *F. temperatum* strains analyzed could produce beauvericin but that none of the strains of *F. subglutinans* could. Unlike the *Fum* cluster, however, the molecular basis for these phenotypic differences was not previously known.

The *Bea* gene cluster contains four genes, *Bea1*-4, of which two, *Bea1* and *Bea2*, are essential for beauvericin production, while the other two, *Bea3* and *Bea4*, encode proteins that repress beauvericin production (21). *Bea4* is not essential for beauvericin production since *F. circinatum* can synthesize beauvericin (5, 38-40), but lacks the gene encoding this protein (21). In fact, deletion of *Bea4* could potentially increase beauvericin production by removing a layer of repressive regulation.

The *Bea1*-encoded non-ribosomal polypeptide (NRPP) synthetase required for biosynthesis of the cyclic depsipeptide beauvericin was first described in the fungus *Beauveria bassiana* over 50 years ago (41, 42) and later confirmed in *F. circinatum*, *F. oxysporum*, *F. proliferatum*, and *F. fujikuroi* (21, 23, 39, 43, 44). Molecular organization of the *Bea* gene cluster has not been analyzed as extensively as has the *Fum* gene cluster. The genomic organization of
the Bea clusters in *F. subglutinans*, *F. temperatum*, *F. circinatum*, *F. proliferatum*, *F. fujikuroi*, *F. mangiferae*, and *F. nygamai* is consistent with respect to gene order, direction of transcription, and genomic context; however, there are differences in individual gene coding sequences.

The available *F. temperatum* genomes are all from beauvericin-producing strains and encode intact, functional sequences for all of the *Bea* genes in the cluster. The *F. subglutinans* genomes all encode functional *Bea2-4* genes. The *Bea1* gene appears to encode a non-functional protein in both of the analyzed sequences from *F. subglutinans*. Both of these genomes contain a 184 bp insertion at position 4,233. This insertion results in a protein projected to be non-functional, whether it alters splicing and intron arrangement or is read as a coding part of the gene. Each strain carries a second, but different, mutation that also inactivates the protein. In RC 298, there is a single nucleotide insertion at position 5,686 that introduces a frame shift resulting in a stop codon 120 bp further downstream (position 5,806) that should prevent translation of a full-length protein. In RC 528, a single nucleotide substitution at position 7,685 results in a premature stop codon 1,907 bp upstream of the 3′ end of the coding region.

The accumulation of only a couple of loss of function mutations in *Bea1* suggests that the process of its inactivation began relatively recently. As both strains have the 184 bp insertion, this genomic change probably occurred first. Assuming this insertion prevents beauvericin accumulation, then subsequent mutations in genes required exclusively for beauvericin biosynthesis would occur without selection acting against them. Thus, the longer a gene has been non-functional, the more mutations it should have accumulated in its coding sequence. After the insertion occurred, flawed transcripts might still produce altered proteins. If so, secondary mutations, such as the *Bea1* single nucleotide insertion or substitution we observed, could have been selected for to reduce the production of proteins with toxic effects or to reduce
the energetic costs due to transcription and translation of non-functional genes, speeding the rate at which mutations accumulate (45-47). Given the difference in secondary mutations seen in the strains sequenced, other strains that do not produce beauvericins could well have other mutations in Bea1, or elsewhere, that prevent beauvericin biosynthesis. Yet the few loss-of function mutants found in either of the two sequenced strains support a recent Bea1 inactivation. Analysis of transcripts from the mutated gene could provide insights into how F. subglutinans has managed the 184 bp insertion in this gene. For example, are the novel introns predicted in the in silico analysis present? Or is the entire insertion translated, which would result in a single base frameshift mutation? The F. temperatum strains that do not produce beauvericin could be of interest as well. Do they carry the 184 bp insertion and either of the other mutations observed in the F. subglutinans genomes? Or is their inability to produce beauvericin due to mutations elsewhere in the Bea cluster or the strains’ genomes?

The nature of the genomic changes that disrupt mycotoxin production play a role in the potential development of diagnostic PCR tests for whether strains could potentially produce fumonisins or beauvericin. Strains of both species would be negative if any primer pairs designed to amplify any portion of the Fum cluster were used, as the entire cluster is missing from the available genomes. A similar test for the potential to produce beauvericin is more problematic. Both species have all of the genes in the Bea cluster, and Bea2-4 are predicted to be intact and functional. Thus, any successful DNA-based assay would need to be specific to Bea1. To detect the aberrant F. subglutinans versions of these genes the assay could have primers that result in a larger fragment due to the 184 bp insertion, or have one primer based on a unique sequence within the inserted region. Tests that detected a secondary SNP or the presence of the insertion also could identify non-functional alleles. Other PCR tests involving Bea1, i.e.,
simply detecting the presence of the gene or a portion of it, would be unable to distinguish a
functional version of the gene from the non-functional version seen in *F. subglutinans*.
Depending on the reason why beauvericin cannot be produced by the three *F. temperatum*
strains, this assay could become even more complex.

In conclusion, we found that 25 strains of *F. subglutinans* and *F. temperatum* from
Argentina could not synthesize fumonisins. The genomic basis for the lack of fumonisin
production is presumably the complete absence of the genes in the *Fum* cluster, given the
available genome sequences. As some *F. temperatum* strains are reported to produce fumonisins
(14, 20), however, sequences of genomes from these strains are needed to understand the
complexities of mycotoxin production in this species. We also confirmed that all tested strains
of *F. subglutinans* and a subset of *F. temperatum* strains cannot synthesize beauvericin, and note
that the lack of beauvericin production cannot be used to definitively identify a strain as *F.
subglutinans*. The *Bea* cluster was organized consistently in terms of location, gene order, and
direction of transcription in *F. circinatum*, *F. fujikuroi*, *F. subglutinans* and *F. temperatum*.
Potential similarities in the *Bea1* sequences from strains of *F. subglutinans* and the non-toxin
producing strains of *F. temperatum* could show whether the initial inactivation event preceded
the separation of *F. subglutinans* and *F. temperatum* as separate species. Since the NRPP
responsible for enniatin synthesis differs in only a few amino acids from the NRPP responsible
for beauvericin synthesis (48), it will be interesting to determine if events that prevent enniatin
synthesis are similar to those that prevent beauvericin synthesis. Our study provides a firm
genetic and physiological base on which future studies of these toxins can be built.

**MATERIALS AND METHODS**
Fungal Isolates. Strains of *Fusarium* were recovered from maize harvested in four regions of Argentina where the presence of *F. subglutinans* and *F. temperatum* had previously been reported (14, 49). Maize grains were incubated on PCNB medium (24) and resulting *Fusarium* colonies were purified by subculturing single microconidia from them. Morphological identifications were made following growth on home made (24) and commercial (Biolife, Milan, Italy) Potato Dextrose Agar (PDA), carnation leaf agar (CLA; 24) and Spezieller Nährstoffarmer agar (SNA; 24) for 10 days at 25°C under 12-hour alternating periods of light (combination of cool white and black lights) and darkness. Colony morphology was evaluated on PDA. Spore morphology was evaluated using spores from colonies growing on CLA or SNA. Strains with the morphological characteristics of *F. subglutinans* described by Leslie and Summerell (24) were selected for DNA-based identification and further study.

DNA-based Identification of Fungal Isolates.

**DNA extraction.** Twenty-five strains with morphology consistent with *F. subglutinans* were selected for DNA-based identification. Isolates were grown on PDA for 2 days at 25°C in the dark. Fresh mycelia were collected by scraping the plate surface and collecting the mycelium in 2-ml tubes. Total genomic DNA was extracted from 30 mg of freeze-dried and ground mycelium by using the Wizard® Magnetic DNA Purification System for Food kit (Promega, Madison, WI) according to the manufacturer’s protocol. DNA was quantified in a NanoDrop spectrophotometer and the DNA concentration adjusted to 20 ng/μl for PCR amplifications.
Gene sequencing. Portions of three housekeeping genes: β-tubulin (Tub2), translation elongation factor (Tef1), and the second largest subunit of RNA polymerase II (Rpb2) were used for species identification. Previously described PCR conditions and primers were used for each gene: BT2a/BT2b for Tub2 (50), EF1/EF2 for Tef1 (51), and 5F/7cR for Rpb2 (52). PCR amplicons were cleaned before sequencing with EXO/FastAp (Exonuclease I, Escherichia coli FastAP thermosensitive alkaline phosphatase, Thermo Fisher Scientific Baltics, Vilnius, Lithuania) to hydrolyze excess primers and nucleotides. Both strands were sequenced with a BigDye™ Terminator v3.1 Cycle Sequencing Ready reaction kit. Sequence reactions were purified by gel filtration through Sephadex G-50 (5%) (Amersham Pharmacia Biotech, Piscataway, NJ), and analyzed on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). The software package Bionumerics 5.1 (Applied Maths, Sint-Martens-Latem, Belgium) was used to align the two DNA strands and edit the sequence. Edited sequences were compared with sequences in the Fusarium-ID (53) and GenBank databases. The phylogenetic species identity of each field strain was assigned to the species of database strains when sequence identity was >98%. NCBI accession numbers for Tef1, Tub2 and Rpb2 sequences for each strain are listed in Table 1.

Phylogenetic analyses. DNA sequences consisting of partial sequences of Tub2, Tef1, and Rpb2 were concatenated and then aligned with ClustalW. The resulting combined data set was analyzed with the Maximum Likelihood algorithm implemented in IQ-TREE (54) with the TNe substitution model (55) and 1000 bootstrap replicates (56). The alignment was deposited in Treebase (https://www.treebase.org/treebase-web/home.html) as accession no. 25,481.
Gene Cluster Analysis

DNA extraction for whole genome sequencing. The genomes of two *F. subglutinans* strains (RC 298 and RC 528) and one *F. temperatum* strain (RC 2914) were sequenced. Each strain was cultivated in 50 ml of complete medium and incubated on an orbital shaker at 150 rpm for 2 days at 25 °C (24). Mycelia were collected following vacuum filtration through non-gauze milk filter disks (KenAG, Ashland, OH) and stored at -20 °C in 2 ml tubes. Frozen mycelia were lyophilized (Labconco Corporation, Kansas City, MO), added to microcentrifuge tubes containing two 4.5 mm zinc-plated steel beads (Daisy BBs, Rogers, AR), and ground to a fine powder in a mixer mill (Verder Scientific, Retsch, Germany). Genomic DNA was isolated by following a modified cetyltrimethyl ammonium bromide (CTAB) protocol (24). The resulting DNA was resuspended in TE (pH 8.0) and stored at -20°C. DNA quality was checked by separation in a 1% agarose gel. DNA concentration was measured with the Quant-iT PicoGreen dsDNA assay kit (Life Technologies, Carlsbad, CA) and the results read in a Synergy H1-hybrid Reader (BioTek Instruments, Inc., Winooski, VT). The DNA was diluted to a final concentration of 100 ng/μl.

Genome sequencing and assembly. Three paired-end libraries (one for each selected strain) were constructed and sequenced with an Illumina MiSeq sequencer using paired-end 300 bp reads at the Kansas State University Integrated Genomics Facility. Genomes were assembled into contigs by using the *de Bruijn* graph-based algorithm implemented in the DISCOVAR *de novo* software from the Broad Institute, Cambridge, MA.
Fastq files were converted to BAM files with the tools in Picard 2.12.1. Though the DISCOVAR de novo assembly does not contain long range scaffolding information, the sequences represented by these fastq files are technically scaffolds due to the presence of some stretches of Ns that bridge small gaps in read coverage. We refer to them as scaffolds, though they are functionally more similar to contigs from other assemblies. Genome sequences were deposited in GenBank-Genomes as JAAIFR000000000 for RC 298, JAAIFQ000000000 for RC 528, and JAAIFN000000000 for RC 2914.

Screening for the presence of beauvericin and fumonisin biosynthetic gene clusters in the newly sequenced genomes of *Fusarium subglutinans* and *Fusarium temperatum*. The newly sequenced genomes *F. subglutinans* (RC 298 and RC 528) and *F. temperatum* (RC 2914) (all three as NCBI Genome submission SUB6672722), as well as the publicly available *F. temperatum* genome CMWF 389 (57), were evaluated for the presence of genes involved in beauvericin and fumonisin production. Genes from the *Bea* cluster in *F. fujikuroi* (FFUJ_09294 to FFUJ_09298) (21) were used as probes in a BLASTn analysis of individual genome sequence databases in CLC Genomics Workbench version 8.0 (CLC Bio-Qiagen, Aarhus, Denmark). Sequences of *Bea* genes from beauvericin-producing strains of *F. circinatum* FSP 34 (58), *F. fujikuroi* IMI 58289 (21, 59), *F. mangiferae* MRC 7560 (21), *F. nygamai* MRC 8546 (60), *F. oxysporum* 4287 (43), *F. proliferatum* NRRL 62905 (21), and beauvericin-non-producing strains of *F. verticillioides* FGSC 7600 (61) and *F. avenaceum* Fa 05001 (48), were identified in GenBank and included in the comparative analysis.
The same BLASTn analysis protocol was used for the *Fum* gene cluster but with the predicted *F. verticillioides* *Fum* gene cluster serving as the reference (FVEG_00316 to FVEG_00329) (27, 62). For the *Fum* cluster, the analysis was extended to regions flanking the cluster, by including the genes described by Proctor et al. (26).

Annotation of the *Bea* biosynthetic genes and *Fum* flanking genes present in the newly sequenced genomes of *F. subglutinans* and *F. temperatum* was done manually, with the gene prediction tools Augustus (63) and FGENESH (64). The location of coding sequences and introns was determined by comparison with the publicly available annotated sequences of the reference strains.

**Genomic context of newly sequenced contigs containing clusters of interest.** The genomic context of the putative *Bea* and *Fum* clusters in the newly sequenced genomes of *F. temperatum* RC 2914 and *F. subglutinans* RC 298 and RC 528 was established. The *F. temperatum* CMWF 389 (57) genome assembly used as a reference is in the scaffolds stage, so dot plots were used to compare these scaffolds with the well-annotated chromosomes of *F. verticillioides* FGSC 7600 (61) and *F. fujikuroi* IMI 58289 (59). The online tool Circoletto (http://tools.bat.infspire.org/circoletto/) was run with default parameters (65). The resulting circular plots provide a global view of the sequence similarity between the *Bea* and *Fum* gene clusters, and flanking regions, from reference genomes and the newly sequenced contigs of *F. subglutinans* and *F. temperatum*. This software also was used to verify that contigs with BLASTn hits contained complete sequences of the clusters of interest, or the flanking regions, and to display aspects of the alignments, such as sequence rearrangements and percent identity.
Beauvericin and fumonisin B$_1$ (FB$_1$) production in vitro. Mycotoxins were produced on PDA, as previously described for *Fusarium* (66). Plates were centrally inoculated with 3 mm diameter mycelial plugs from the edges of 7-day-old SNA cultures. Inoculated plates were incubated for 15 days in darkness at 25°C. Each plate was inoculated in duplicate. This experiment was performed once.

Chemicals and preparation of standards. All solvents (HPLC grade) were purchased from VWR International SRL (Milan, Italy). Ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA). Beauvericin standards (purity > 99%) were purchased from Sigma-Aldrich (Milan, Italy) and FB$_1$ from Biopure (Romer Labs Diagnostic GmbH, Getzersdorf, Austria). Standard stock solutions (1 mg/mL) were prepared by dissolving the solid commercial toxin standards in methanol. For working solutions of beauvericin, some of the methanol stock solution was dried under a nitrogen stream at 50°C and reconstituted with methanol-water (70:30, v/v). Standard solutions for UPLC calibration were prepared by using different concentrations in a range of 0.02-40.00 µg/mL. Working stock solutions of FB$_1$ were prepared by drying some of the stock solution under a nitrogen stream and reconstituting it with acetonitrile-water (1:1, v/v). Standard solutions for UPLC calibration were prepared by using different concentrations in a range of 0.01-1.00 µg/mL. Standard solutions were stored at -20°C and warmed to room temperature (~ 20-22°C) prior to use.
Determination and confirmation of beauvericin production. Ten grams of culture material were extracted with 15 mL of methanol on an orbital shaker (150 rpm) for 30 min. Six mL of the extract were evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 1.5 mL of methanol-water (70:30, v/v) and filtered through a 0.2 µm regenerated cellulose filter (Grace Davison Discovery Science, Columbia, MD). Ten µL of the extract were injected into the full loop injection system of a Waters Acquity UPLC® system (Milford, MA), equipped with an ESI interface, and with a binary solvent manager, a sample manager, a column heater, a photodiode array and QDa detectors. The analytical column was an Acquity UPLC® BEH C18 (2.10 × 100 mm, 1.7 µm) preceded by an Acquity UPLC® in-line filter (0.20 µm). The temperature of the column was set at 50°C. The flow rate of the mobile phase was set at 0.35 mL/min. The toxins were determined in both detectors, i.e., the photodiode array set at 205 nm, and, after the effluent, into the ESI interface, without splitting. The mobile phase consisted of a gradient with two components: (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The initial composition 50:50, A:B) was kept constant for 2 min, then solvent B was increased linearly to 75% in 8 min, followed by another linear increase to 80% in 2 min and, finally, kept constant for 4 min. For column re-equilibration, solvent B was linearly decreased to 50% in 1 min and then kept constant for 4 min. The limit of quantification (LOQ) of the method was 0.01 µg/kg.

For LC/MS analyses, the ESI interface was used in positive ion mode, with the following settings: desolvation temperature 600°C; capillary voltage 0.80 kV, sampling rate 5 Hz. The mass spectrometer was operated in full scan (600-800 m/z) and in single ion recording (SIR) mode, by monitoring the individual mass of beauvericin: 784 m/z; elemental formula [M + H]+:C45H57N3O9. MassLynx® 4.1 mass spectrometry software was used for data acquisition and
processing. The retention time for beauvericin was ~ 9.80 min. Beauvericin was quantified by measuring peak areas and comparing these values with a calibration curve obtained from standard solutions (48, 67, 68).

**Determination and confirmation of fumonisin production.** Ten grams of culture material were extracted with 15 mL of methanol - water (70:30, v/v) on an orbital shaker (150 rpm) for 60 min. Six mL of the extract were evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 1.5 mL of acetonitrile:water (30:70, v/v), filtered with RC 0.2 μm filters (Phenomenex, Torrance, CA), derivatized, as described below, and quantified by HPLC/FLD. To derivatize a sample, 50 μL of a sample extract was mixed with 50 μL of o-phtaldialdehyde (OPA) by shaking for 50 s in the HPLC autosampler of an Agilent 1100 equipped with a binary pump and a column thermostat set at 30°C. The 100 μL volume was injected by full loop 3 min after adding the OPA reagent for fumonisin analysis. The analytical column was a Symmetry Shield RP18 (4.6 × 150 mm, 5 μm, Waters, Milford, MA) with a guard column inlet filter (0.5 μm × 3 mm diam., Postnova Analytics Inc., Salt Lake City, UT). The mobile phase consisted of a binary gradient whose initial composition was 57% A (water:acetic acid, 99:1, v/v) / 43% B (acetonitrile:acetic acid, 99:1, v/v) and kept constant for 5 min. Solvent B was then linearly increased to 54% at 21 min, linearly increased again to 58% at 25 min, and finally kept constant for 5 min. The flow rate of the mobile phase was 0.80 mL/min. The fluorometric detector was set at wavelengths: ex = 335 nm and emm = 440 nm. Retention time for FB1 was 17 min. The LOQ of the method was 0.01 μg/kg.

Fumonisin B1 was confirmed by UPLC® with an Acquity QDa mass detector. The chromatographic separation was performed on an Acquity UPLC® BEH C18 column (2.1 × 100
mm, 1.7 µm) preceded by an Acquity UPLC® in-line filter (0.2 µm). The temperature of the column was set at 50°C. The flow rate of the mobile phase was set at 0.4 mL/min. Solvent A was water, and solvent B was methanol, both containing 0.1% acetic acid. A gradient elution was used beginning with 90% of A and 10% of B. The gradient changed (i) from 10% to 50% of solvent B in 10 min, (ii) was kept constant for 4 min, (iii) was linearly increased to 90% of solvent B in 3 min, and finally (iv) kept constant for 4 min. For column re-equilibration, solvent B was decreased to 10% in 1 min and kept constant for 3 min.

For LC/MS analyses, the ESI interface was used in positive ion mode, with the following settings: desolvation temperature 600°C; capillary voltage 0.80 kV, sampling rate 5 Hz. The mass spectrometer was operated in full scan (100 - 800 m/z) and in single ion recording (SIR) mode, by monitoring the individual mass (FB₁ 722.40 m/z). Retention time for FB₁ was 16 min. Empower™ 2 Software (Waters, Milford, MA) was used for data acquisition and processing. The LOQ was 0.01 µg/mL for FB₁ (48, 67, 68).

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| Strain   | Geographic Origin | Beauvericin Production | NCBI sequence number |
|----------|-------------------|------------------------|---------------------|
|          |                   |                        | Tef1 | Tub2 | Rpb2 |
| **F. temperatum** |                 |                        |      |      |      |
| ITEM 16196 | Belgium           | ND                     | MT345561 | MT345559 | MT345560 |
| RC 1164    | Tartagal          | +                      | MT337672 | MT337622 | MT337647 |
| RC 1189    | Tartagal          | +                      | MT337676 | MT337626 | MT337651 |
| RC 1199    | Tartagal          | +                      | MT337669 | MT337619 | MT337644 |
| RC 1369    | NOA 1             | +                      | MT337677 | MT337627 | MT337652 |
| RC 1494    | NOA1              | -                      | MT337673 | MT337623 | MT337648 |
| RC 1520    | NOA1              | -                      | MT337674 | MT337624 | MT337649 |
| RC 1677    | SEBA              | +                      | MT337679 | MT337629 | MT337654 |
| RC 1780    | NOA1              | -                      | MT337678 | MT337628 | MT337653 |
| RC 1789    | NOA1              | +                      | MT337675 | MT337625 | MT337650 |
| RC 2881    | NOA1              | +                      | MT337670 | MT337620 | MT337645 |
| RC 2914    | NOA1              | +                      | MT337668 | MT337618 | MT337643 |
| RC 2977    | NOA1              | +                      | MT337671 | MT337621 | MT337646 |
| **F. subglutinans** |               |                        |      |      |      |
| NRRL 22016 | USA               | ND                     | HM057336 | U34417 | JX171599 |
| RC 298     | SEBA              | -                      | MT337655 | MT337605 | MT337630 |
| RC 528     | Lajitas           | -                      | MT337657 | MT337607 | MT337632 |
| RC 1047    | SEBA              | -                      | MT337661 | MT337611 | MT337636 |

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| Strain  | Geographic Origin | Beauvericin Production | NCBI sequence number |
|---------|-------------------|------------------------|----------------------|
| RC 1096 | SEBA              | -                      | MT337662 MT337612 MT337637 |
| RC 1098 | SEBA              | -                      | MT337663 MT337613 MT337638 |
| RC 1594 | SEBA              | -                      | MT337659 MT337609 MT337634 |
| RC 1655 | SEBA              | -                      | MT337656 MT337606 MT337631 |
| RC 1739 | SEBA              | -                      | MT337660 MT337610 MT337635 |
| RC 1986 | SEBA              | -                      | MT337658 MT337608 MT337633 |
| RC 2491 | Lajitas           | -                      | MT337667 MT337617 MT337642 |
| RC 2535 | Lajitas           | -                      | MT337666 MT337616 MT337641 |
| RC 2548 | Lajitas           | -                      | MT337664 MT337614 MT337639 |
| RC 2620 | Lajitas           | -                      | MT337665 MT337615 MT337640 |

*No strain produced fumonisin when cultured on PDA. The SEBA region contains three locations in south-east Buenos Aires province, with a 13.9°C (8.2-20.2°C) mean annual temperature and 550-900 mm annual precipitation. Tartagal and Lajitas are locations in the Salta province, with a 21.1°C (14.3-26.4°C) mean annual temperature and 650-800 mm annual precipitation and 20.4°C (16.7-28.1°C) mean annual temperature and 500-800 mm annual precipitation, respectively. NOA1 contains four locations across Quebrada de Humahuaca in the Jujuy province, and with an 11.7°C (5.1-16.3°C) mean annual temperature and 400 mm annual precipitation.

cRange: 7-400 μg/kg; mean production: 71 μg/kg; median production: 11 μg/kg.

dND – No Data from this study.
|                  | F. temperatum RC 2914 | F. subglutinans RC 298 | F. subglutinans RC 528 |
|------------------|------------------------|------------------------|------------------------|
| Scaffold N50 (bp)| 334,266                | 228,189                | 203,510                |
| Total number of scaffolds | 720                     | 4,088                  | 1,418                  |
| Longest scaffold (bp) | 1,464,565              | 974,989                | 997,454                |
| Total bases in 1 kb+ scaffolds | 43,206,368            | 50,560,826            | 43,931,993            |
| Total bases in 10 kb+ scaffolds | 42,527,692         | 49,665,874            | 43,037,708            |
| Genomic read fold coverage | 53.3                  | 100.7                  | 60.9                  |
Figure captions

**FIG 1** Phylogenetic tree derived from combined DNA sequences of *Tub2, Tef1*, and *Rpb2*. The evolutionary history was inferred using the Maximum Likelihood method. Numbers on branches indicate bootstrap values based on 1000 pseudoreplicates. RC strains are from the strain collection at the National University of Rio Cuarto; ITEM strains are from ISPA, Bari, Italy; NRRL strains are from the USDA-ARS Culture Collection at the National Center for Agricultural Utilization Research, Peoria, Illinois.

**FIG 2** Comparison between Chromosome 9 of *Fusarium fujikuroi* IMI 58289 (NC_036630.1) and Scaffold 7 of *Fusarium temperatum* CMWF 389 (LJGR01000007.1). Dot plot alignments show good synteny across both sequences, but also some inverted regions and gaps.

**FIG 3** Comparison between Chromosome 1 of *Fusarium verticillioides* FGSC 7600 (NC_031675.1) and Scaffolds 1 and 12 of *Fusarium temperatum* CMWF 389 (LJGR01000001.1 and LJGR01000012.1). Dot plot alignments show that both, scaffolds 1 and 12 almost completely cover Chromosome 1. Dot plot alignments show good synteny across sequences, but also some inverted regions and gaps.

**FIG 4** Circos plots showing the synteny across *Bea* (left) and *Fum* (right) clusters, a chromosome segment from the *F. temperatum* reference, and contigs from the new genome assemblies. Ribbons connecting the sequences represent local alignments produced by the BLAST algorithm. The ribbon colors indicate percentage identity, with blue (<50%), green...
(<75%), orange (<99%) and red (≥99%). A. Ideogram built using the Circoletto program comparing sequences of the Bea cluster of *F. fujikuroi* IMI 58289 (segment that protrudes at the upper right of the circle) with the new sequenced genomes of *F. subglutinans* RC 298, RC 528, *F. temperatum* RC 2914, and the South African reference strain CMWF 389 (sections of the circle in dark grey). Each section represents sequence from an individual strain. B. Ideogram built using the Circoletto program showing a comparison between the Fum cluster and related flanking regions (5' flanking region: ZNF1, ZBD1; 3' flanking region: ORF20, ORF21) of *F. verticillioides* FGSC 7600 (segment that protrudes at the upper right of the circle) and new sequenced genomes of *F. subglutinans* RC 298 and *F. temperatum* RC 2914 (sections of the circle in dark grey). In order to show the absence of the Fum cluster and the adjacency between the flanking regions in greater detail, only one strain of each species is included in the graph. In both *F. subglutinans* and *F. temperatum* the Fum cluster 5' and 3' flanking regions are directly adjacent in their respective contigs, indicating the absence of the Fum cluster. Note twists in the ribbons here, indicating inverted orientations of multiple segments of these flanking regions.

**FIG 5** Organization of the Bea gene cluster and flanking genes. The arrows represent the indicated genes while the direction of the arrow shows direction of transcription. Blue arrows indicate known Bea cluster genes (21). Grey arrows indicate genes that flank the Bea cluster. Genes A, B, C, D, F, G, H, I, J, K, L, M, N, and O share >70% identity with FFUJ_09292, FFUJ_09293, FFUJ_09297, FFUJ_09299, FFUJ_09291, FFUJ_09286, FFUJ_09287, FNYG_14765, FNYG_14764, FOXG_11842, FOXG_11843, FOXG_11844, FFUJ_08099, and FFUJ_08100, respectively. Genes E, P, and Q share <50% identity with FFUJ_09300, FOZG_00061, and FPRN_10819, respectively. Ψ – Pseudogene (non-functional). Strains used
are: *Fusarium avenaceum* Fa 05001, *Fusarium circinatum* FSP 34, *Fusarium fujikuroi* IMI 58289, *Fusarium mangiferae* MRC 7560, *Fusarium nygamai* MRC 8546, *Fusarium oxysporum* 4287, *Fusarium proliferatum* NRRL 62905, *Fusarium subglutinans* RC 298, *Fusarium temperatum* RC 2914, and *Fusarium verticillioides* FGSC 7600.

**FIG 6** A) Single base mutations in RC 298 and RC 528 that could contribute to a non-functional *Bea1* (NRPP) gene. B) Presence of the 184 bp insertion in both *F. subglutinans* genomes analysed in this study. Red squares indicate genomic locations in the alignment where the indicated polymorphisms are observed.

**FIG 7** Organization of genes flanking the *Fum* cluster. The genes and the different genomic contexts (GC1, GC2, GC3) were previously described by Proctor et al. (26). The *Fum* cluster is in different chromosomal locations in GC1, GC2 and GC3. Arrows represent the indicated genes while the direction of the arrow shows the direction of transcription. Only the marginal genes (*Fum19* and *Fum21*) of the *Fum* cluster are shown. Genes A, B, C, D, E, F, G, H, I, J, K, L, M, and N share >70% identity in BLASTp analysis with FVEG_00333, FVEG_00334, FVEG_00312, FVEG_00311, FFUJ_09236, FFUJ_09237, FFUJ_09258, FFUJ_09259, FVEG_10515, FFUJ_12036, FVEG_10524, FVEG_10525, FFUJ_12035, and FOXB_15017, respectively. The strains examined in this study are *Fusarium circinatum* FSP 34, *Fusarium fujikuroi* IMI 58289 (GCA_900079805), *Fusarium proliferatum* NRRL 62905 (GCA_900029915), *Fusarium subglutinans* RC 298, *Fusarium temperatum* RC 2914, and *Fusarium verticillioides* FGSC 7600 (GCA_000149555).
